IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT:	G. Scott Herron
APPLICATION NO.:	09/905,704
FILING DATE:	February 27, 2001
TITLE:	IN VIVO ASSAY FOR ANTI-ANGIOGENIC COMPOUNDS
EXAMINER:	Valerie E. Bertoglio
GROUP ART UNIT:	1632
ATTY. DKT. NO.:	23946-08185

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Mail Stop Petition, Commissioner For Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below:

Dated: February 23, 2004 By: _____
Jennifer R. Johnson, Reg. No. 50,784

MAIL STOP PETITION
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PETITION FOR LATE ENTRY OF PRIORITY CLAIM AND PRIORITY DOCUMENTS UNDER 37 C.F.R. § 1.55(a)

SIR:

1. Applicant petitions for entry of the following accompanying papers with respect to the priority claim in this case being made after Notice of Allowance dated December 22, 2003.

Copies of the applications from which priority is claimed:

Country:	United States
Application No.:	60/271,778
Filing Date:	February 27, 2001

Country:	Patent Cooperation Treaty
Application No.:	WO 00/56988
Filing Date:	March 24, 2000

Application WO 00/56988 claims priority from:

Country:	United States
Application No.:	60/126,015
Filing Date:	March 24, 1999

2. The following reference is required by 35 U.S.C. § 119, 120 and 37 C.F.R. § 1.78(a)(2), 1.78(a)(5):

This application claims priority under 35 U.S.C. §119(e) from United States Provisional Application Serial No. 60/271,778, filed February 27, 2001, and is a continuation-in-part of and claims priority from WO 00/56988, filed March 24, 2000 titled "Immortal Microvascular Endothelial Cells And Uses Thereof," which claims priority to the United States Provisional Application Serial No. 60/126,015, filed March 24, 1999.

3. Applicant declares that the entire delay between June 27, 2001, the date the claim was due under 37 C.F.R. § 1.78(a)(2) & 1.78(a)(5), and the date of this petition was unintentional.

4. Attached is a check in the amount of $1,430.00 including a $1,300.00 surcharge for late entry (37 C.F.R. § 1.17(t)) and a $130.00 petition fee (37 C.F.R. § 1.17(i)).

Respectfully submitted,
G. SCOTT HERRON

Dated: February 23, 2004

By: _____
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Rev. 10/95

U.S. Department of Commerce
Patent and Trademark Office

TRANSMITTAL FORM

(to be used for all correspondence during pendency of filed application)

Application Number	**09/905,704**
Filing Date	**February 27, 2001**
First Named Inventor	**G. Scott Herron**
Group Art Unit Number	**1632**
Examiner Name	**Valerie E. Bertoglio**
Total Number of Pages in This Submission: **98**	Attorney Docket Number: **23946-08185**

ENCLOSURES *(check all that apply)*

- ☒ Fee Transmittal Form (in duplicate)
  - ☒ Check Enclosed
- ☒ Return Receipt Postcard
- ☐ Response to Notice to File Missing Parts
- ☐ Assignment & Recordation Cover Sheet
- ☐ Declaration
- ☐ Power of Attorney
- ☐ Application Data Sheet
- ☐ Information Disclosure Statement & PTO/SB/08A
  - ☐ Copies of IDS Cited References
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- ☒ Revocation and Substitute Power of Attorney
- ☐ Issue Fee Transmittal
- ☐ Letter to Chief Draftsperson
- ☐ Formal Drawing(s):
  - [ ] Sheet(s) of Figure(s) [ ]
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- ☐ Certified Copy of Priority Document(s)
- ☐ After Allowance Communication to Group
- ☒ Petition for Late Entry of Priority Claim and Priority Documents Under 37 C.F.R. §1.55(a)
- ☒ Copy of Application for US 60/271,778 (29 pgs)
- ☒ Copy of Application for WO 00/56898 (65 pgs)
- ☐ _____
- ☐ _____
- ☐ _____

REMARKS:

SIGNATURE OF ATTORNEY OR AGENT

Signature:			
Attorney/Reg. No.:	**Jennifer R. Johnson, Reg. No. 50,784**	Dated:	**February 23, 2004**

CERTIFICATE OF MAILING

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Signature:			
Typed or Printed Name:	**Jennifer R. Johnson**	Dated:	**February 23 2004**
Express Mail Mailing Number *(optional)*:			

FEE TRANSMITTAL for FY 2004

Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT | **$ 1,430.00**

Complete if Known	
Application Number	09/905,704
Filing Date	February 27, 2001
First Named Inventor	G. Scott Herron
Examiner Name	Valerie E. Bertoglio
Art Unit	1632
Attorney Docket No.	23946-08185

METHOD OF PAYMENT (check all that apply)

☒ Check ☐ Credit Card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account:

Deposit Account Number: 19-2555

Deposit Account Name: Fenwick & West LLP

The Commissioner is authorized to: *(check all that apply)*

☐ Charge fee(s) indicated below ☒ Credit any overpayments

☒ Charge all required fee(s) or any underpayment of fee(s) due under 37 CFR §1.16 or §1.17 during the pendency of this application

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to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	

SUBTOTAL (1) ($)

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below		Fee Paid
Total Claims		-20**=		X =	
Independent Claims		-3** =		X =	
Multiple Dependent				=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	**Reissue independent claims over original patent
1205	18	2205	9	**Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner actio	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	130.00
1807	50	1807	50	Processing fee under 37 CFR 1.17(g)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	
Other fee (specify) Extension for Late Entry of Prior Claim					1300.00

SUBTOTAL (3) $ 1,430.00

*Reduced by Basic Filing Fee Paid

SUBMITTED BY

Name (Print/Type)	Jennifer R. Johnson	Registration No. (Attorney/Agent)	50,784	Complete (if applicable) Telephone: (650) 335-7213
Signature		Date		February 23, 2004

IMMORTAL MICROVASCULAR ENDOTHELIAL CELLS AND USES THEREOF

Technical Field

The present invention relates to immortal microvascular endothelial cells having normal karyotype that demonstrate resistance to apoptosis, methods for producing said cells, and methods of use thereof.

Background of the Invention

Emerging evidence suggests that apoptosis and the cell cycle are closely linked and use parts of the same molecular machinery (Meikarnatz W, Schlegel R. Apoptosis and the cell cycle. Journal of Cellular Biochemistry 1995, 58(2):160-74; King K.L., Cidlowski J.A.. Cell cycle and apoptosis: common pathways to life and death. Journal of Cellular Biochemistry 1995, 58(2):17580; Kasten M, Giordano A. pRb and the Cdks in apoptosis and the cell cycle. Cell Death and Differentiation 1998, Review:132-140). Cells progressing through the cell cycle become more susceptible to apoptosis versus quiescent cells but interestingly, cell cycle arrest in late G1 or S phase potentiates apoptosis. Cell cycle checkpoint proteins (e.g. p53, pRB and cyclin dependent kinase inhibitors, p21 and p27) are involved in making cell fate decisions of apoptosis or cycle arrest but precise mechanisms remain unclear (Evan G, Littlewood T. A matter of life and cell death. Science 1998, 281(5381):1317-22). It is known that unrepaired DNA and chromosomal damage triggers apoptotic induction justifying these checkpoint proteins as "guardians of the genome" (Lane D.P. Cancer. p53, guardian of the genome Nature 1992, 358(6381):15-6).

Chromosomal damage in the form of telomeric DNA shortening during cell division may serve as a "biological clock" that triggers replicative senescence. Cell cycle arrest at senescence is a complex and as yet poorly defined process that involves genetic programming much like the differentiated phenotype. Telomeric DNA shortens at a certain rate during each cell division due to the inability of standard

DNA polymerases to synthesize DNA at the ends of chromosomes. Once a certain length is reached, a sensor determines that it's time for the cell to senesce and stop dividing. (Harley C, Vaziri H, Counter D, et al. The telomere hypothesis of cellular aging. Exp Gerontol 1992, 27:375-382; Sedivy JM. Can ends justify the means?: telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 1998, 95(16):9078-81). There are many pathways that lead to the final common state of replicative senescence but DNA damage is recognized as a major path involving p53-mediated G1 arrest (Di Leonardo A, Linke SP, Clarkin K, et al. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes and Development 1994, 8(21):2540-51). The state of replicative senescence is considered an "activated" state by many investigators, particularly with regards to the expression of genes involved in extracellular matrix metabolism (West MD. The cellular and molecular biology of skin aging. Archives of Dermatology 1994, 130(1):87-95; Campisi J, Dimri GP, Nehlin JO, et al. Coming of age in culture. Experimental Gerontology 1996, 31(1-2):7-12).

During angiogenesis EC proliferation occurs in an area proximal to the tip of new vessel and these vessels represent sprouting postcapillary venules (Folkman J, Bream H. Angiogensis and inflammation. In. (second 4 ed.) (JI Gallin LG, and R. Snyderman, ed. New York: Raven Press Ltd., 1992. In Inflammation: Basic Principles and Clinical Correlates). The EC cell cycle can be arrested by three main mechanisms: 1) Growth factor removal; 2) Extracellular matrix signaling a "nonpermissive" environment (Ingber DE. Extracellular matrix as a solid-state regulator in angiogenesis: identification of new targets for anti-cancer therapy. Seminars in Cancer Biology 1992, 3(2):57-63) and; 3) Contact inhibition.

An emerging theme in the control of EC proliferation is that growth factor and ECM signaling are tightly coupled via matricellular proteins such that endogenous angiostatic factors appear to sequester growth factors, block receptor activation and even induce EC apoptosis (Bornstein P. Diversity of function is inherent in

matricellular proteins: an appraisal of thrombospondin 1. Journal of Cell Biology 1995, 130(3):503-6; Sage EH. Pieces of eight, bioactive fragments of extracellular proteins as regulators of angiogenesis. Trends Cells Biol 1997, 7:182-186; Kupprion C, Motamed K, Sage EH. SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. Journal of Biological Chemistry 1998, 273(45):29635-40; Lucas R, Holmgren L, Garcia I, et al. Multiple Forms of Angiostatin Induce Apoptosis in Endothelial Cells. Blood 1998, 92(12):4730-4741). EC cell-cell signaling is an equally strong regulator of EC proliferation *in vitro* and signaling through intercellular contacts clearly regulates EC cell cycle machinery (Yoshizumi M, Lee WS, Hsieh CM, et al. Disappearance of cyclin A correlates with permanent withdrawal of cardiomyocytes from the cell cycle in human and rat hearts. Journal of Clinical Investigation 1995, 95(5):2275-80; Nakamura Y. Cleaning up on beta-catenin [news]. Nature Medicine 1997, 3(5):499-500).

The mechanisms of EC cell cycle arrest and induction of the quiescent state by the above three events are different but reversible. Clearly none represent replicative senescence. Thus, while many angiostatic factors work by G1 growth arrest and delay of entry into S phase (Funk SEea). Differential effects of SPARC and15(2):363-72; Baldin V, Roman AM, Bosc-Bierne I, et al. Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. Embo Journal 1990, 9(5):1511-7; Hori A, Ikeyama S, Sudo K. Suppression of cyclin D1 mRNA expression by the angiogenesis inhibitor TNP-470 (AGM-1470) in vascular endothelial cells. Biochemical and Biophysical Research Communications 1994, 204(3):1067-73; Abe J, Zhou W, Takuwa N, et al. A fumagillin derivative angiogenesis inhibitor, AGM-1470, inhibits activation of cyclin-dependent kinases and phosphorylation of retinoblastoma gene product but not protein tyrosyl phosphorylation or protooncogene expression in vascular endothelial cells. Cancer Research 1994, 54(13):3407-12) molecular mechanism(s) responsible for EC senescence are not known.

The adult endothelium *in vivo* is remarkably quiescent and endothelial cells (EC) divide very slowly unless activated in some way. Isolation and culture of EC result in a semi-activated state in which cells retain some specialized characteristics but lose others (Cines DB. Glycoprotein IIb/IIIa antagonists: potential induction and detection of drug-dependent antiplatelet antibodies. American Heart Journal 1998, 135(5 Pt 2 Su):S152-9). All human EC appear to retain the ability to divide many times *in vitro* but their continued survival depends on a variety of different factors and conditions (Bicknell R. Endothelial Cell Culture. (Bicknell R, ed. Oxford: Cambridge University Press, 1996). When EC become activated via inflammatory cytokines, oxidative stress or other pathologic insults many different vasoprotective genes are induced. Some of these include; Bcl-2 family members, A20, MnSOD, 70-kDa HSP, heme oxygenase-1, and VEGF. Without the induction of such genes EC survival and replication would not match the loss of EC during states of inflammation.

Like all somatic cells, human EC undergo replicative senescence after a finite number of divisions which varies between 20 and 50 population doublings (PD) depending on the tissue of origin and culture conditions. Human vascular endothelial cells (HUVECs) appear to respond to autocrine production of IL-1 by undergoing senescence (Maier JA, Voulalas P, Roeder D, et al. Extension of the life-span of human endothelial cells by an interleukin-1 alpha antisense oligomer. Science 1990, 249(4976):1570-4; Maier JA, Statuto M, Ragnotti G. Endogenous interleukin 1 alpha must be transported to the nucleus to exert its activity in human endothelial cells. Molecular and Cellular Biology 1994, 14(3):1845-51). More recently, VEGF was found to both delay the onset of replicative senescence in HDMEC and reverse senescence of HDMEC when added to cultures grown without VEGF (Watanabe Y, Lee SW, Detmar M, et al. Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) delays and induces escape from senescence in human dermal microvascular endothelial cells. Oncogene 1997, 14(17):2025-32). Senescent HDMEC expressed high levels of both p16 and p21 CDK inhibitors relative to VEGF-treated HDMEC and VEGF withdrawal increased p16 with little effect on p21. The

data suggests that replicative senescence in HDMEC is associated with G1 growth arrest involving p16.

VEGF is known to be an important survival factor for all EC and its absence has dramatically negative effects on vasculogenesis, angiogenesis and vascular remodeling (Risau W. Mechanisms of angiogenesis. Nature 1997, 386:671-674). VEGF signaling pathways depend on a set of receptor tyrosine kinases (nearly) unique to EC; flt-1/VEGFR1, flk-1/KDR/VEGFR2 and TIE-2. VEGFR2 downregulation is thought to be important in EC senescence and death *in vitro* (Hewett PW, Murray re O, Rafii S, Ely S, et al. Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. Nature 1998, 394(6693):588-92). The other 95% of uninfected primary EC also bypassed replicative senescence as "bystanders" in mass cultures because VEGFR2 expression was induced by the paracrine effect of KSHV-infected EC.

In the skin, both epidermal and mesenchymal cells express VEGF; however, VEGF is also an autocrine factor synthesized and secreted directly by HDMEC in response to hypoxia (Detmar M, Brown LF, Berse B, et al. Hypoxia regulates the expression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and its receptors in human skin. Journal of Investigative Dermatology 1997, 108(3):263-8).

There are a number of barriers to the development of rapid and reproducible human microvascular remodeling assay systems that the art has failed to address. These include cell variability; cell viability; and morphogenetic response measurement.

Cell variability: Primary microvascular endothelial cell (MEC) cultures isolated from human tissues represent mixtures of cells (i.e. mass cell cultures) derived from both arteriolar and venular microvessels 15-50um in diameter, as well as, lymphatic vessels. When these cells are expanded in culture, a variable number of different cell populations will predominate according to purity of the cells, donor age and source, growth conditions and passage number. Primarily, it is this cell population variability that gives rise to markedly different responses to morphogenetic stimuli

and resultant irreproducibility in standard *in vitro* angiogenic assay systems (e.g. 3D collagen and Matrigel).

Cell viability: Primary human MEC, as defined above (and further within), have finite lifespans *in vitro* which vary between 20 and 40 population doublings. Depending on culture conditions MEC will undergo either "culture senescence" or "replicative senescence" wherein cultures cannot be expanded any longer and stop dividing. Culture senescence is seen when suboptimal growth conditions result in growth factor unresponsiveness and/or apoptosis due to a variety of different conditions (e.g. pC02, p02, serum concentration, survival factor concentration, lack of flow, etc), whereas, MEC replicative senescence is observed when telomeric DNA shortens to such a degree that the senescence program is activated and cells also become growth factor unresponsive. It is this finite lifespan that limits the development of angiogenic assay systems because MEC survival cannot be maintained long enough to allow statistically significant and reproducible measurements of the process.

Morphogenetic response measurement: The formation of new capillaries in permissive extracellular matrices and tissues is complex and not well understood. Visualization of the angiogenic process requires image analysis of vessel formation inside these matrices. Rapid and efficient methods for quantifying this process either *in vitro* or *in vivo* are rudimentary and consist of primarily of micromorphometric measurements of vessel lumens in stained tissue sections. This process is slow and tedious. One reason for this is that human MEC have not been genetically "tagged" to stably express marker elements that can be tracked, traced or otherwise detected so as to produce images that can be digitally converted (e.g. by immunofluoresence microscopy) into usable information. Software programs measuring this process are not readily available or have not been developed.

The present invention, as hereinbelow illustrates, provides new methods and compositions for overcoming these obtacles.

Summary of the Invention

The present invention provides methods for the generation of immortal microvascular endothelial cells, including immortal human dermal microvascular endothelial cells (HDMECs), having normal karyotype that demonstrate resistance to apoptosis. The immortal microvascular endothelial cells of the present invention are not transformed, and have no activated oncogenes (i.e., that result in malignant transformation). The cells have an essentially normal phenotype as compared to primary microvascular endothelial cells. These immortal cells were generated by the introduction of the human telomerase reverse transcriptase catalytic subunit gene (hTERT) into primary endothelial cells. Endothelial cells from other human tissue locations and other animal sources may also be produced by the methods of the present invention.

In one embodiment, the present invention includes a composition of immortal microvascular endothelial cells, where the cells of the compositions each contain a recombinant expression cassette encoding telomerase. The expression cassette can include a number of control elements. Typically the expression cassette contains at least a promoter operably linked to the telomerase coding sequence. The immortal microvascular endothelial cells of the present invention (a) have a normal karyotype, (b) are resistant to apoptosis relative to primary microvascular endothelial cells, and (c) are not transformed. Further, the immortal microvascular endothelial cells have an essentially normal phenotype relative to primary microvascular endothelial cells.

In a preferred embodiment of the present invention the immortal microvascular endothelial cells are derived from primary human dermal microvascular endothelial cells.

In still another preferred embodiment, the present invention provides immortal microvascular endothelial cells that incorporate a genetic label or tag. A further preferred embodiement the present invention provides immortal microvascular endothelial cells that incorporate eGFP producing a uniform population of fluorescently labeled cells. One aspect of the present invention is a composition of microvascular endothelial cells that demonstrate superior survival characteristics both

in vitro and *in vivo* relative to primary cells. More specifically such superior survival includes an extended cellular life span as well as resistance to apoptosis comparable to young primary human dermal microvascular endothelial cells.

In one aspect of the invention, the immortal microvascular endothelial cells can be used to generate xenograft mice. Such mice provide an angiogenesis model useful, for example, for screening therapeutic compounds.

Further, the immortal microvascular endothelial cells can be used to generate new blood vessels, reline the surfaces of existing vasculature, create new vasculature and vascular structures in subjects. Therapeutic uses of these cells include, treatment, for example, of atherosclerosis. The cells are also useful in methods of reversing vascular system inflammatory response.

In addition, the immortal microvascular endothelial cells of the present invention provide methods of treating tumors, e.g., administering immortal àsing human EC (e.g., by incorporation of immortal microvascular endothelial cells into animal models of angiogenesis and vascular remodeling).

Still a futher aspect of the present invention provides *in vivo* human microvascular remodeling assay systems using eGFP-labeled immortalized microvascular endoethelial cells which form fluorescent capillary blood vessels and vessel density which can be assessed by digital imaging.

The immortal microvascular endothelial cells of the present invention also provide a number of *in vivo* therapeutic strategies, including, but not limited to, the following: 1) replacement cells in disease states involving inadequate or dysfunctional proliferation/regression of host EC at the site of disease via transplantation; 2) gene transfer vehicles to express ectopic genes requiring vascular delivery in monogenetic diseases and autoimmune diseases; and 3) gene delivery vehicles to express ectopic genes (e.g., angiostatic factors; AS, ES, TSP, TIMPs) that would deter the proliferation and spread of occult malignant tumors during the early stages of tumor-induced angiogenesis.

The immortal microvascular endothelial cells of the present invention have characteristics that are useful in the design of vascular model systems and therapeutic strategies for treating age-related diseases of the vasculature.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Citation of the documents herein is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents. Further, all documents referred to throughout this application are hereby incorporated in their entirety by reference herein.

Brief Description of Figures

Figures 1A to 1F are photomicrographs of transfection results obtained using the LZRS-eGFP vector in HDMEC.

Figure 2 presents a graph that shows an inverse correlation of reporter gene expression efficiency and passage number.

Figures 3A and 3B present results of transfection of HDMEC with the LZRS-vector carrying coding sequences for the CD34 protein.

Figures 4A to 4D present the results of fluorescence activated cell sorting that was carried out on transfected cells.

Figure 5 presents a vector construct containing hTERT coding sequences.

Figure 6 presents the results of RT-PCR analysis to test for the presence of the hTERT transgene.

Figure 7 presents Southern blotting results where the hybridization was carried out with a biotinylated telomere probe.

Figure 8 presents karyotypic analysis for hTERT (+) HDMEC.

Figures 9A and 9B presents the results of FACs quantification of the expression of Apo2.7, an early and specific mitochondrial-associated apoptotic marker.

Figures 10A and 10B presents the results of the effect of population doubling number (PD#) on absolute baseline of (unstimulated) values of apoptosis using both nuclear and mitochondrial apoptotic analysis.

Figures 11A and 11B presents the results of stimulated apoptosis analyses, as described for Figures 10A and 10B, that were repeated on separate cells.

Figure 12 presents the results of a series of immunoblots using polyclonal anti-human ES antiserum (Fibroblast and microvascular endothelial cell endostatin).

Figure 13 presents the results of a series of immunoblots using polyclonal anti-human ES antiserum (microvascular endothelial cell endostatin).

Fig 14; Presents data demonstrating the relative apopoptic resistance of hTERT(+)HDMEC induced by permissive 3D collagen matrix exposure.

Fig 15; Presents data showing the utility of using eGFP-labeled hTERT(+)HDMEC for tracking morphogenetic patterns of cells forming microvascular structures *in vitro*.

Fig 16; Presents data demonstrating the utility of using eGFP-labeled hTERT(+)HDMEC in 3D Matrigel for visualizing microvessel formation.

Fig 17; Presents an example of how hTERT(+)HDMEC can be used to test the angiostatic characteristics of cyclo-oxygenase inhibitor compounds.

Fig 18; Presents the results of a human *in vivo* microvascular remodeling assay system in which SCID-mice are implanted with Matrigel mixed with hTERT(+)HDMEC. Results show creation of SCID-human chimeric blood vessels.

Fig 19; Presents data demonstrating the presence of fluorescent human blood vessels in the SCID mouse and the superiority of hTERT(+)HDMEC versus primary cells.

Fig 20; Presents data showing how human Type 4 collagen immunoreactivity is used to estimate human microvascular density. Results demonstrate the superiority of hTERT(+)HDMEC at *in vivo* microvessel formation.

Fig 21; Presents immuno-micromorphometry data in graphic format showing quantification of microvessel density and demonstration of the superiority of hTERT(+)HDMEC at *in vivo* microvessel formation.

Fig 22; Presents data demonstrating the specificity of hTERT(+)HDMEC at *in vivo* microvessel formation. HT1080 fibrosarcoma cells and primary dermal fibroblasts exhibit no human Type 4 collagen immunoreactivity.

Fig 23; Presents data demonstrating the specificity of hTERT(+)HDMEC at *in vivo* microvessel formation using eGFP-labeled HT1080 human fibrosarcoma cells, human embryonic kidney 293 cells and hTERT(+)HDMEC.

Fig 24; Presents data on the effect of phorbol ester (PMA) and anti-vitronectin receptor antibody (LM609) on eGFP-labeled hTERT(+)HDMEC using the *in vivo* microvascular remodeling assay system.

Fig 25; Presents data on microvessel density using anti-human Type 4 collagen immuno-reactivity comparing the effect of phorbol ester (PMA) and anti-vitronectin receptor antibody (LM609) on vessel formation *in vivo*.

Fig. 26; Presents data on microvessel density using anti-human Type 4 collagen immuno-reactivity comparing the effect of b-FGF and VEGF on vessel formation *in vivo*.

Fig. 27; Presents quantitative immuno-micromorphometry data in graphic format comparing the effects of b-FGF, VEGF, LM609 and PMA on microvessel density *in vivo*.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional methods of molecular biology, chemistry, biochemistry and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., *Remington's Pharmaceutical Sciences*, 19th Edition (Easton, Pennsylvania: Mack Publishing Company, 1995); *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); Wang, A.M., *et al.* in *PCR Protocols: a Guide to Methods and Applications* (M.A. Innis, *et al.*, eds.) Academic Press (1990); Kawasaki, E.S., *et al.*, in *PCR Technology: Principles and Applications of DNA Amplification* (H.A. Erlich, ed.) Stockton Press (1989); Hochuli, E., in *Genetic Engineering, Principals and Practice, Vol. 12* (J. Stelow Ed.) Plenum, NY,

pp. 87-98 (1990); Ausubel, F.M., *et al.*, *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., Media PA; and, Sambrook, J., necting ascending arterioles and descending venules, and; d) deep horizontal vascular plexus. Primary human dermal microvascular endothelial cells (HDMEC) can be derived, for example, from neonatal foreskin.

"Inflammatory response" as used herein, refers to a nonspecific defensive reaction of the body to invasion by a foreign substance or organism that involves phagocytosis by white blood cells and is often accompanied by accumulation os pus and an increase in the local temperature.

"Atherosclerosis" as used herein, refers to deposition of lipid with proliferation of fibrous connective tissue cells in the inner walls of the arteries.

"Angiostatic Switch" as used herein, refers to the induction of endothelial cell (EC) apoptosis by angiostatic factors in actively growing blood vessels.

"Resists cell death" as used herein, refers to a first cell line that, when compared to another cell line(s) of the same type, does not enter senescence, apoptosis, or cell-cycle arrest.

"Finite replicative life"as used herein, refers to a finite number of times the DNA of a cell can be replicated, typically corresponds to a finite number of cell divisions that a cell may undergo.

"Immortal" as used herein, refers to cells that do not enter into "replicative senescence." Typically, the cells are capable of dividing at least twice as many times as those from which they they were derived (parental). Unlike transformed cells, immortal cells are supposed to maintain the normal phenotype, karyotype and function of parental cells.

"Mitotic Clock" as used herein, refers to chromosomal damage in the form of telomeric DNA shortening during cell division may serve as a "biological clock" that triggers replicative senescence. Cell cycle arrest at senescence is a complex and as yet poorly defined process that involves genetic programming much like the diffferentiated phenotype (Harley C, Vaziri H, Counter C, et al. The telomere hypothesis of cellular aging. Exp Gerontol 1992, 27:375-382; Sedivy JM. Can Ends

Justify The Means?: telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 1998, 95(16):9078-81). There are many pathways that lead to the final common state of replicative senescence but DNA damage is recognized as a major path involving p53-mediated G1 arrest (Di Leonardo A, Linke SP, Clarkin K, et al. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes and Development 1994, 8(21):2540-51). The state of replicative senescence is considered an "activated" state by many investigators, particularly with regards to the expression of genes involved in extracellular matrix metabolism (West MD. The cellular and molecular biology of skin aging. Archives of Dermatology 1994, 130(1):87-95; Campisi J, Dimri GP, Nehlin JO, et al. Coming of age in culture. Experimental Gerontology 1996, 31(1-2):7-12).

"Nucleic acid expression vector" or "Expression cassette" refers to an assembly which is capable of directing the expression of a sequence or gene of interest. The nucleic acid expression vector includes a promoter which is operably linked to the sequences or gene(s) of interest. Other control elements may be present as well. Expression cassettes described herein may be contained within, for example, a plasmid or viral vector construct. In addition to the components of the expression cassette, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA *(e.g.,* a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (e.g., a SV40 or adenovirus origin of replication).

By "subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys anntroduce one or more exogenous DNA

moieties into suitable host cells. The term refers to both stable and transient uptake of the genetic material, and includes uptake of peptide- or antibody-linked DNAs.

A "vector" is capable of transferring gene sequences to target cells (e.g., viral vectors, non-viral vectors, particulate carriers, and liposomes). Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

A "selectable marker" or "reporter marker" refers to a nucleotide sequence included in a gene transfer vector that has no therapeutic activity, but rather is included to allow for simpler preparation, manufacturing, characterization or testing of the gene transfer vector.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual in a formulation or composition without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

II. Modes of Carrying Out the Invention

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

1. A Rapid and Efficient Transgene Delivery System for Human Dermal Microvascular Endothelial Cells.

Gene transfer into vascular endothelial cells (EC) has presented particular problems. Generally low bacterial plasmid transfection efficiencies ranging between 0.1-20% are typical (Sun B, Plumpton C, Sinclair JH, et al. In vitro expression of calcitonin gene-related peptide in human endothelial cells transfected with plasmid and retroviral vectors. Neuropeptides 1994, 26(3):167-73; Tanner FC, Carr DP, Nabel GJ, et al. Transfection of human endothelial cells. Cardiovascular Research 1997, 35(3):522-8; Fife K, Bower M, Cooper RG, et al. Endothelial Cell Transfection With Cationic Liposomes and Herpes Simplex Thymidine Kinase Mediated Killing. Gene Therapy 1998, 5(5):614-620). Experiments performed in support of the present invention have demonstrated that using retroviral systems in which the vector remains episomal (Nabel EG, Nabel GJ. Complex models for the study of gene function in cardiovascular biology. Annual Review of Physiology 1994, 56(1):741-61) results in high efficiency gene transfer (> 70%) to primary human dermal microvascular endothelial cells (HDMEC). Enhanced green fluorescent protein (eGFP; Chalfie M et al., Science, 1994, 263:802-5; Clontech Laboratories Inc., Palo Alto, California) was used as a reporter when early passage, b-FGF-stimulated cells were used (Romero L et al., Journal of Cellular Physiology, 1997, 173:84-92). Photomicrographs of transfection results obtained using the LZRS-eGFP vector (Deng H et al., Nature Biotechnology, 1997, 15:1388-91; Paul Khavari, Department of Dermatology Stanford University) in HDMEC are shown in Figures 1A to 1F. In the figures, Figure 1A and 1B are primary HDMEC without retroviral infection, Figures 1C and 1D are primary HDMEC without treatment, and Figures 1E and 1F are the same cells treated for 24hr with bFGF.

The graph shown in Figure 2 shows an inverse correlation of reporter gene expression efficiency and passage number. Importantly, these genetically-modified HDMEC continue eGFP expression for up to four weeks in culture, display HDMEC marker expression, respond to inflammatory cytokines and 3D collagen similar to unmodified, control HDMEC.

Other genes of importance in HDMEC biology have been expressed using this expression system with high efficiency of gene transfer. One of these genes is CD34. Results of transfection of HDMEC with the LZRS-vector carrying coding sequences for the CD34 protein (Romero, et al., 1997 J. Cell Physiol 173:84-92) are presented in Figures 3A and 3B. Immunofluorescence staining of cells was carried out using standard procedÃâå éoâëåßÑÅåÉnal method of developing an *in vivo* model to incorporate the hTERT(+)HDMEC involves implantation of a Matrigel cushion containing bFGF into the ventral subcutaneous tissue of a SCID mouse followed by intralesional injection of hTERT(+)HDMEC after 3 days. This methods utilizes the teachings of the present invention and the methods of Tony Passaniti, Ph.D. (University of Maryland, Greenebaum Cancer Center, Bressler Research Building, Baltimore).

The approach just described, i.e. creation of endothelialized dermal equivalents, could properly be termed "vasculogenesis" because EC first form vascular tubes and lumens from the clustering, realignment and remodeling of mixtures of dermal cells within the matrix. This process is clearly different from the intussusceptive formation of tubules in response to 3D collagen or Matrigel or the sprouting of vessels from pre-existing capillaries, "angiogenesis." It is likely that the hTERT(+)HDMEC mass cell cultures of the present invention, derived from pools of human neonatal tissue, may contain a subpopulation of "de-differentiated angioblast-like" EC that could support vasculogenic growth if these cells have a survival advantage. Further, during routine purifications of HDMEC, the PECAM(-) population of cells is often discarded. This population typically represent mixtures of dermal fibroblasts, myofibroblasts, pericytes, dermal dendricytes and other uncharacterized spindle cells. These PCAM(-) cell populations may be useful in the preparation of the endothelialized dermal equivalent because they may be enriched in perivascular cells that are involved in stabilization and morphogenetic patterning of newly formed capillaries. Furthermore, perivascular cells may also be transduced with the hTERT expression vector (described in Example 1), characterized by FACS, and used to recreate a dermal environment that is far more durable than repopulation with primary cells.

9.2 Effect of *fas* Expression

Fas belongs to the TNFR (TNF Receptor) and signals via the death domain in its cytoplasmic tail. HDMEC do not express Fas at baseline and are not susceptible to FasL-induced apoptosis. Fas mRNA expression can be induced by incubation of normal HDMEC with plasma from patients with TTP (thrombotic thrombocytopenia purpura) and such treatment leads to HDMEC apoptosis which can be blocked by soluble anti-Fas antibody (Laurence J, Mitra D, Steiner M, et al. Plasma From Patients With Idiopathic and Human Immunodeficiency Virus-Associated Thrombotic Thrombocytopenic Purpura Induces Apoptosis in Microvascular Endothelial Cells. Blood 1996, 87(8):3245-3254; Mitra D, Jaffe EA, Weksler B, et al. Thrombotic thrombocytopenic purpura and sporadic hemolytic-uremic syndrome plasmas induce apoptosis in restricted lineages of human microvascular endothelial cells. Blood 1997, 89(4):1224-34). A recombinant Fas/anti-Fas IgG system has been employed to induce apoptosis in both human keratinocytes and dermal fibroblasts (Freiberg RA, Spencer DM, Choate KA, et al. Specific triggering of the Fas signal transduction pathway in normal human keratinocytes. Journal of Biological Chemistry 1996, 271(49):31666-9; Freiberg RA, Spencer DM, Choate KA, et al. Fas signal transduction triggers either proliferation or apoptosis in human fibroblasts. Journal of Investigative Dermatology 1997, 108(2):215-9). This system will be used to induce apoptosis in the hTERT(+)HDMECs of the present invention after incorporation into the *in vivo* model, described above. This system offers the advantage of triggering apoptosis without simultaneous activation of survival signals and/or DNA repair mechanisms inherent to apoptotic induction by TNF , LPS and UV light. Furthermore, the system is specific because anti-Fas IgG treatment induces death only in cells overexpressing Fas and is inducible, since in the absence of anti-Fas IgG, Fas does not multimerize and activate death.

hTERT(+)HDMECs are transduced with LZRS-Fas expressing the full length Fas driven by the retroviral LTR and first tested *in vitro* to determine if they are more or less susceptible to apoptosis versus primary controls with and without triggering

death with CH-11, a Fas cross-linking antibody (Freiberg RA, Spencer DM, Choate KA, et al. Specific triggering of the Fas signal transduction pathway in normal human keratinocytes. Journal of Biological Chemistry 1996, 271(49):31666-9). Next these hTERT(+)HDMECs are incorporated into the organotypic dermal equivalent and grafted onto SCID mice. Fas expression levels are checked by IF, before and after Fas transduction, *in vitro* and *in vivo* using the same antibody. Finally, CH-11 is administered by intradermal injection near the graft site to trigger apoptosis *in vivo*. Apoptosis is measured *in vitro* and by using TUNEL staining *in vivo*. The *in vitro* method uses FACS methodology and is performed essentially as follows. Cells are treated with TNFα + actinomycin D (AMD), LPS + cycloheximide (CHX), or UV light, 16 hours prior to the experiments. Also, one group of cells is serum starved for 40 hours. The cells are incubated with primary antibody (Apo2.7 IgG, Immunotech) + PE-conjugated secondary antibody at 37°C. The data is collected and analyzed with CellQuest (Becton Dickinson) or Coulter EPICS cell sorter.

The *in vitro* nuclear fragmentation method is performed essentially as follows. The cells are cultured in 48-well plates and confluent for 2 days before the assay. The reagents and protocol were from Boehringer Mannheim. Briefly, the plates are centrifuged for 10 minutes at 200 g and the supernatants are removed. The cells are then lysed for 30 minutes at room temperature. The plates are re-centrifuged and one tenth of the supernatant is used for the incubation with anti -histon-biotin and anti-DNA-POD. After 2 hours incubation, the plates are washed three times and incuated with substrate solution. The absorbance is measured at 405 nm with microplate reader (BioRad).

The TUNEL assay is performed essentially as follows. The percentage of apoptotic cells are detected by the APO-BRDU terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling assay (Gavrieli, et al., J. Cell Biol. 119: 493-501) according to manufacturer's instructions (Phoenix Flow Systems, Phoenix, AZ).

The hTERT(+)HDMECs will likely exhibit a resistance to Fas-triggered death relative to primary HDMEC *in vitro* but undergo apoptosis *in vivo* and thus serve as an inducible apoptosis model system.

10. Uses and Applications of the hTERT(+)HDMEC Cells and Methods of the Present Invention

The present invention provides methods for the generation of immortal human dermal microvascular endothelial cells (HDMECs), having normal karyotype that are resistant to apoptosis. The hTERT(+)HDMEC cells are not transformed, and have no activated oncogenes (i.e., that result in malignant transformation). The cells have an essential normal phenotype as compared to primary HDMECs. These immortal cells were generated by the introduction of the human telomerase reverse transcriptase catalytic subunit gene (hTERT) into primary HDMEC. No oncogenes were used to generate the immortalized cells of the present invention. These cells have been designated hTERT(+)HDMECs.

The hTERT(+)HDMECs of the present invention have many commercial, screening, and therapeutic applications. As described above, the cells can be used to generate xenograft mice to provide an angiogenesis model useful for, e.g., screening therapeutic compounds. The cells also provides means to identify compounds that will affect expression of telomerase in HDMEC. The cells can also be used to screen compounds that facilitate or block the formation of new blood vessels.

Further, the hTERT(+)HDMECs can be used to generate new blood vessels, reline the surfaces of existing vasculature, create new vasculature and vascular structures, in subjects by injection of the cells to, for example, a site of interest. Therapeutic uses of these cells include, treatment, for example, of atherosclerosis. As blood vessels age they change how they are presented to the immune system, the hTERT(+)HDMECs of the present invention can be used to restore the vasculature and retain normal presentation to the immune system (for example, by relining arteries of the heart). The cells are also useful in methods of reversing vascular system inflammatory response.

In addition, the hTERT(+)HDMECs of the present invention provide methods of treating tumors, increasing blood flow to tumors by administering hTERT(+)HDMEC, and by increasing blood flow into tumors improve the administration of anti-tumor and/or therapeutic compounds.

Further, following the guidance of the present specification, hTERT(+)HDMEC can be created from several different human anatomic sites. In the same way, hTERT(+)EC can be created from animals, different animal anatomic sites, or from genetically-modified (e.g. transgenic) animals. The hTERT(+)EC of the present invention can be supplied as a commercial product that provides EC which are easy to grow, have a normal karyotype, display a consistent phenotype, are not transformed, and are immortal. The hTERT(+)ECs of the present invention provide the means to obtain large quantities of genetic material (e.g. for gene microarray studies) and proteins (e.g. for extracellular matrix studies).

hTERT(+)EC of the present invention can be obtained from a number of human and animal sources including, but not limited to, the following: normal neonatal foreskin, adult normal skin, and pediatric skin; as well as, adult pathologic skin derived from patients with different cutaneous disease states (including but not limited to, scleroderma, psoriasis, Epidermolysis Bullosa, hemangiomas and other vascular proliferative lesions, skin tumors, vasculitic lesions, nonhealing wounds and wounds in different stages of healing). By the methods of the present invention such cell types are established, and characterized at the molecular level (e.g gene expression differences as determined by micro-array technology) to determine which genes are up or down regulated and whether undiscovered genes are expressed by distinct strains. Importantly, these genes and their gene products can then be tracked in the *in vivo/in situ* state, providing a link between various strains *in vitro* and their anatomic locations in the skin. This information provides investigators with details about what makes a certain vascular disease attack just one type of vessel and not another (e.g., in leukocytoclastic vasculitis) and facilitates the development of more effective and specific therapies. Creation of hTERT(+)HDMEC lines from malignant tumor-induced angiogenic vessels allows a molecular analysis of the differences

between these vessels and neovascularized tissues in wounds and other skin pathologies.

The hTERT(+)EC of the present invention also provide pharmacologic and toxicologic methods of screening and testing new drugs designed to modulate the growth of blood vessels *in vivo* using human EC (e.g., by incorporation of hTERT(+)HDMEC into animal models of angiogenesis and vascular remodeling). Also, hTERT(+)HDMEC derived from pathologic tissues can be incorporated into these model systems to evaluate their potential for forming new blood vessels or to influence the regression of others.

The hTERT(+)HDMEC of the present invention provide a number of *in vivo* therapeutic strategies, including, but not limited to, the following: 1) syngeneic/autografted hTERT(+)EC can be used as replacement cells in disease states involving inadequate or dysfunctional proliferation/regression of host EC at the site of disease via transplantation (e.g., scleroderma, keloid scars, atherosclerotic plaques, venous or arterial ulcers, diabetic vasculopathy, flap-graft sites in plastic surgery and other healing wounds with poor vascularization, etc); 2) syngeneic/autografted hTERT(+)EC can be used as gene transfer vehicles to express ectopic genes requiring vascular delivery in monogenetic diseases (hemophilia, thalasemia, cystic fibrosis, hypercholesterolemia, etc.), and autoimmune diseases (diabetes, thyroiditis, etc.); and 3) syngeneic/autografted hTERT-EC can be used as gene delivery vehicles to express ectopic genes (angiostatic factors; AS, ES, TSP, TIMPs) that would deter the proliferation and spread of occult malignant tumors during the early stages of tumor-induced angiogenesis.

Further, new evidence suggests that adult vascular tissue and/or bone marrow contains undifferentiated "white blood cells" that represent precursors to mature, differentiated EC (Asahara T, Murohara T, Sullivan A, et al., Isolation of putative progenitor endothelial cells for angiogenesis, Science 1997;275(5302):964-7.; Shi Q, Rafii S, Wu MH, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92(2):362-7). Human adult and/or neonatal HDMEC may contain small subpopulations of these angioblastic EC precursors. Accordingly, the

hTERT(+)HDMEC strains of the present invention may contain a continuously dividing, immortalized subpopulation of cells with this property and such cells can be used in the applications described above.

The experiments described herein demonstrated that microvascular endothelial cells can be effectively immortalized by hTERT alone in the absence of malignant transformation. In addition, the results described herein showed that hTERT immortalized EC exhibited functional and morphogenetic characteristics of parental cells. These hTERT(+)EC lines also display a survival advantage beyond the hurdling of replicative senescence as they appear to be more resistant to programmed cell death. Such characteristics are useful in the design of vascular model systems and therapeutic strategies for treating age-related diseases of the vasculature.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the compositions and methods of the present invention, and are not intended to limit the scope of what the inventor regards as the invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Experimental

The following preparations and examples are given to enable those skilled in the art to more clearly understand and practice the present invention. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

Example 1

Microvascular Endothelial Cell Preparation, Transgene Delivery System, and Transfection Method for HDMEC

A. Establishment of Endothelial Cell Culture

Human dermal microvascular endothelial cells (HDMEC) for making the TERT-1 cell line were purchased from Clonetics (San Diego, CA). Primary HDMEC (designated "lab-made") for making TERT-2 and TERT-3 cell lines were obtained directly from neonatal foreskin samples.

Isolation and growth of primary neonatal HDMEC was performed as described by Romero, et al. (Romero, LI, Zhang, DN, Herron, GS and Karasek, MA. IL-1 Induces Major Phenotypic Changes in Human Skin Microvascular Endothelial Cells. J. Cell. Physiol. 1998; 173:84-92). Briefly, neonatal foreskin tissue obtained from Stanford University School of Medicine Labor and Delivery ward were stored less than 2 days in Hank's balanced salt solution. Tissue was sectioned into 5 mm^2 pieces and incubated overnight at 4°C in 50 caseolytic U/ml of dispase (Collaborative Research, Bedford, MA) in HBSS, pH 7.2. The epidermis was gently separated from dermis and the gentle, outward pressure was applied to release the microvasculature from dermis into the medium. The cells were centrifuged for 5 minutes at 1000xg, plated into a 25 cm tissue culture flask pre-coated with 1% gelatin for every two foreskins and called Passage zero (P0). Growth media were EBM-2 MV BulletKit or EBM (Clonetics).

When P_0 cells reached 80-100% confluency, they were trypsinized, mixed with anti-PECAM IgG-coated beads (Sigma, St. Louis, MO) in the ratio of 5-10 beads/cell and incubated for 30 minutes at room temperature. The cells bound to beads were recovered with a magnetic particle concentrator (Promega, Madison, WI), plated in a new gelatin-coated 25 cm flask and referred to PECAM(+) passage 1 (P1) cells. At confluency they were replated in a gelatin-coated 6-well cluster dishes for retrovirus infection

B. Gene Transduction

Gene transfer was achieved by retrovirus-mediated gene transfer. A retroviral vector was used to transduce hTERT genes to the HDMEC: LZRS-hTERT. LZRS-hTERT was constructed by Eco-R1 digestion of pGRN145 (Geron Corp) followed by subcloning into the LZRS plasmid (Kinsella T, Nolan G. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther* 1996; **7**:1405-1413). Orientation and correct sequence of the full length hTERT cDNA in LZRS was confirmed by complete DNA sequence analysis. Retroviral DNA was transfected into the Phoenix 293 amphotropic retroviral packaging cell system, and puromycin (2.5 _g/ml) was added to the culture 3 days after transfection. Packaging cells were cultured in puromycin-supplemented 10% fetal bovine serum medium until confluency, switched to medium without puromycin and incubated at 32°C overnight. Retrovirus was collected into 15 ml polystyrene tubes and centrifuged at 300xg for 10 minutes to remove contaminated cells before they were stored at -80°C.

Viral titers determined by the infection of NIH-3T3 cells were estimated at approximately $5X10^6$/ml. Retroviral infection of HDMEC was performed as follows. On infection day, cells were incubated briefly in growth medium supplemented with 5 _g/ml polybrene for 10 minutes. The medium was then replaced with 2.5 ml of polybrene supplemented retroviral supernatant. Plates were centrifuged at 300xg at 32° C for 1 hour followed by incubation at 32° C for 5 additional hours.

The expression of hTERT in the retroviral vector was driven by Moloney murine leukemia virus 5' long terminal repeat (5'-LTR) promoter.

C. Telomeric Repeat Amplification Protocol (TRAP) Assay

Either a standard protocol (Kim and Wu, Nucleic Acids Research 25(13):2505-2597(1997)) or a PCR-ELISA based protocol (Boehringer Mannheim) was used to measure the telomerase activity from the hTERT transgene. Typically, the PCR-ELISA method was for HDMEC. For visualizing the DNA ladder with the standard protocol, 1000 or 5000 cell equivalents were analyzed. For PCR-ELISA

assay, 2000 cell equivalents were used. The PCR-ELISA protocol was provided by the assay kit manufacturer (Boehringer Mannheim).

A quantitative PCR-ELISA TRAP assay showed that telomerase activity of hTERT(+) HDMEC was maintained for over 100 PDs and the level of telomerase activity achieved in hTERT(+)HDMEC was comparable to that expressed by the 293 human embryonic kidney tumor cell line. By contrast, parental HDMEC expressed endogenous telomerase transiently at early PD. Using RT-PCR, the presence of hTERT RNA transcribed from the transduced retroviral vector at PD60 in HDMEC was also confirmed.

Further, different parental EC strains were shown to senesce at different PD, exhibiting typical flattened cell morphology and senescence-associated (SA) beta-galactosidase activity between PD 19-60. By contrast, none of the hTERT(+)HDMEC lines showed significant SA-beta gal activity and morphologically appeared similar to early passage, proliferating primary EC. Thus, the ectopic expression of hTERT in EC extended the replicative lifespan of all EC strains examined to over twice that of primary EC, technically defining these TERT-EC lines as immortalized (Shay, JW, Wright, WE, Werbin, H., Defining the molecular mechanisms of human cell immortalization. Biochem. Biophys. Acta 1991; 1072:1-7).

D. Telomere Length Assay

Isolation of genomic DNA and development of mean TRF (telomere restriction fragment) Southerns were performed according to published procedures (Harley, et al., Nature 345: 458, 1990; Allsopp, RP, et al., PNAS 89: 10114, 1992; and Vaziri et al., Am J Hum Genet. 52:661, 1993). Briefly, the genomic DNA (3 g) was digested with *Hinf* I/*Rsa* I and run on 0.6% agarose gel. The gel was transferred onto a positively charged nylon membrane, which was hybridized at 65°C overnight. Signal on the membrane was detected by chemiluminescence. Calculation of mean TRF length followed a standardized procedure (Levy, M. et al. J Mol Biol. 225:951, 1992).

To examine the effects of hTERT expression on EC telomeres, telomere lengths were assessed by telomere restriction fragment (TRF) Southern analysis. The change in telomere length was evaluated for several representative hTERT(+)HDMEC clones as a function of PD. Telomere shortening was observed for these clones up to PD 100-120 followed by consistent stabilization at approximately 4.5-5 kbp by PD 100-120.

E. RT-PCR for Telomerase Transcripts

Primers for RT-PCR were as follows:

i) in hTERT gene-- sense: CACCTCACCCACGCGAAAA; and
anti-sense: CCAAAGAGTTTGCGACGCATGTT;

ii) at the border of hTERT and retroviral LZRS sequence--
sense: TCCTGAAAGCCAAGAACGCA; and
anti-sense: GACCAACTGGTAATGGTAGCGA.

The sample RNA was isolated by using TRIZOL (Gibco). The RT-PCR was performed by a one-step RT-PCR system (Gibco).

F. Flow Cytometry

The antibodies used for flow Cytometry were anti- PECAM (Becton Dickinson), ICAM (Pharmingen), and Apo2.7 (Immunotech). Some cells for measuring ICAM were stimulated with TNF (100 ng/ml) for 15 hours before the assay. For apo 2.7 assay, the cells were treated with TNF + actinomycin D (AMD), LPS + cycloheximide (CHX), or UV light 16 hours prior to the experiments. One group of cells were serum starved for 40 hours. The cells are incubated with either PE conjugated primary antibody or un-conjugated primary antibody + PE-conjugated secondary antibody at 37^{0}C. The data was collected and analyzed with CellQuest (Becton Dickinson) or Coulter EPICS cell sorter.

Parental HDMEC and hTERT(+)HDMEC lines showed high PECAM-1 reactivity (Fig.4B). Expression of von Willebrand factor and LDL uptake also showed no differences between parental and hTERT(+)HDMEC lines. Basal and TNF -

stimulated cell surface expression of ICAM-1, VCAM-1 and E-selectin were similar in both parental and hTERT(+)HDMEC lines. The data showed that hTERT(+)HDMEC lines continuously passaged *in vitro* over twice the normal replicative lifespan of primary EC exhibit both the functional and differentiated phenotype of early passage, primary EC.

G. Tubule Formation on Matrigel or Following 3d Collagen Overlay

i) Tubule formation on Matrigel. Matrigel (Collaborative Biomedical Products) was placed on ice and allowed to thaw overnight in a darkened cold room or refrigerator. Three hundred fifty microliters of the Matrigel was layered onto a pre-chilled well of a 6-well plate and then placed in an incubator at 37° C for one half hour for the Matrigel to solidify. About 350,000 cells in M199/15% FBS/10 U/ml heparin/16 g/ml ECGF were then seeded onto the matrix and allowed to incubate at 37°C, 5% CO2 environment.

ii) Collagen overlay. Primary HDMEC or hTERT(+)HDMEC were overlaid with a 1:1 mixture of Vitrogen 100 (Celtrix) and 2X Iscoves (Gibco). Adding a small amount of NaOH to the mixture brought the color back to the original of 2X Iscoves. Solidification of the collagen gel occurred within 30 min., followed by incubation at 37°C. Plates were photographed at 8 and 24 hour using the Zeiss inverted microscope.

Morphogenetic responses were evaluated by exposing parental HDMEC and hTERT(+)HDMEC (TERT-1, TERT-2, and TERT-3) cell lines to 3D type I collagen. Both early passage parental and hTERT-bearing HDMEC cell populations (at all passage numbers tested) responded similarly by efficiently forming"angiogenic webs;" late passage, senescent HDMEC did not form such webs. Further, senescent HDMEC did not form tubules in 3D collagen. The TERT-1 cell line did not form tubules well in 3D collagen. Similar responses were seen for the cell lines when tested for their response to Matrigel. A commercial source of EC was used to prepare TERT-1, whereas, TERT-2 and TERT-3 were derived from a pool of freshly obtained primary neonatal EC.

H. Cell Death ELISA Assay

Cells to be tested (including primary HDMEC and hTERT(+)HDMEC lines) were cultured in 48-well plates and maintained at confluency for 2 days before the assay. The reagents and protocol, Cell Death ELISA Assay kit, were from Boehringer Mannheim. Briefly, the plates were centrifuged for 10 minutes at 200 g and the supernatants were removed. The cells were then lysed for 30 minutes at room temperature. The plates were re-centrifuged and one tenth of the supernatant was used for the incubation with anti -histon-biotin and anti-DNA-POD. After 2 hours of incubation, the plates were washed three times and incubated with substrate solution. The absorbance was measured at 405 nm with microplate reader (BioRad).

The basal apoptotic rate was monitored in HDMEC and it was found that both early and late passage parental HDMEC showed lower nuclear fragmentation relative to mid passage HDMEC with differences reaching statistical significance for PD15 vs both PD5 and PD25. The effect of PD on apoptosis in primary HDMEC cultures was verified by FACS analysis of Apo 2.7 expression, an apoptotic-specific mitochondrial protein. Two different hTERT(+)HDMEC cell lines, TERT-1 (PD 60, 70, 80) and TERT-3 (PD50, 80), showed results comparable to early and late passage parental HDMEC.

Apoptosis was also evaluated after stimulation with several different EC apoptotic inducers using two hTERT(+)HDMEC lines (TERT-1 and TERT-3) and late passage, presenescent parental HDMEC as controls. Four different conditions for inducing EC apoptosis all showed the same result that hTERT(+)HDMEC resisted apoptotic induction relative to primary HDMEC. Except for TNF + AMD induction in TERT-1 cells, both hTERT(+)HDMEC lines expressed statistically significant lower nuclear fragmentation versus controls in response to all treatments. LPS + CHX induction showed significantly decreased Apo2.7 expression in TERT-1 vs control, whereas, other treatments did not reach statistical significance. The TERT-3 line that exhibited lower baseline apoptosis generally showed the lowest stimulated apoptotic

rates. UV light-induced nuclear fragmentation and Apo2.7 expression appeared to reveal the most dramatic differences between primary and both TERT-1 and -3 lines.

I. Growth Patterns and Karyotype Analysis

The growth patterns of hTERT-EC lines were compared and no significant differences in their growth rates compared to parental EC were seen. hTERT(+)HDMEC lines showed contact inhibition and exhibited normal pRB phosphorylation patterns in response to serum deprivation and hydroxyurea-induced cell cycle arrest. Furthermore, none of the hTERT(+)HDMEC lines formed colonies in soft agar.

Following mitotic arrest with Colcemid®, monolayer cell culture in log-phase growth were harvested by standard cytogenetic methods of trypsin dispersal, hypotonic shock with 0.075 M KCl, and fixation with 3:1 methanol/acetic acid fixative (Barch, M. J., T. Knutsen, et al., Eds. (1997). The AGT cytogenetics laboratory manual. New York, Lippincott-Raven). Mitotic cells slide preparations were analyzed by the GTW banding method (Seabright, M. (1971). "A rapid banding technique for human chromosomes." Lancet 2: 971-972).

G-banding and cytogenetic analyses showed parental HDMEC have a normal diploid karyotype which was maintained upon immortalization by introduction of hTERT. Taken together, these results indicate that introduction of telomerase into normal human EC does not lead to abnormal growth patterns, cell transformation, or genomic instability.

The results presented above show, the general applicability of using ectopic expression of hTERT to bypass replicative senescence while maintaining EC phenotypic and morphogenetic characteristics *in vitro*. Upon stable transfection or retroviral transduction of hTERT, telomerase activity was detectable in all EC and telomere lengths decreased with time in culture and then stabilized. To date, hTERT(+)HDMEC lines of the present invention, both clones and mass cultures, have achieved PDs (PD60-130) over twice that of parental or control vector transduced

cells (PD30 – 50) and therefore are considered "immortal" (Shay, JW, Wright, WE, Werbin, H. Defining the molecular mechanisms of human cell immortalization. Biochem. Biophys. Acta 1991; 1072:1-7). The hTERT(+)HDMEC lines of the present invention have been continuously passaged without evidence of altered morphology or changes in growth patterns.

Example 2

Evaluation of Expression of Vasoprotective Factors (VEGF and NO)

A. VEGF Analysis

VEGF transcript and protein expression are analyzed according to the following methods including ELISA and semiquantitative RT-PCR. A number of different hTERT containing EC cell lines, including the cell lines described herein, are screened at several population doubling points (e.g., PD30, 60 and 90, versus vessel-matched primary EC cultures at senescence. First, the cell lines are evaluated using the ELISA assay. If major increases or decreases in VEGF concentrations are observed, transcript analysis is performed via semiquantitative RT-PCR on representative cell lines. In the event of the observation of consistent patterns, representative cell lines are compared to primary EC at early and mid PD.

RNA Isolation

Total RNA is isolated from cultured HDMEC and hTERT(+)HDMEC using the Trizol method (Gibco BRL), according to the manufacturer's procedure and then stored at -80 _C until use.

RT- PCR and Semi-Quantitative PCR

The primers for PCR are as follows:

GAPDH sense (5'-AATCCCATCACCATCTTCCA-3'), and
antisense (5'-GTCATCATATTTGGCAGGTT-3') oligonucleotides;
VEGF sense (5'-CCATGAACTTTCTGCTGTCTT-3'), and
antisense (5'-ATCGCATCAGGGGCACACAG-3'), oligonucleotides.

The amplification products are predicted to be 558 bp for GAPDH, and 249 bp for VEGF. The VEGF primers are chosen in exon 1 and exon 3 of the VEGF gene resulting in a PCR product of 294 bp irrespective of the splice-form produced.

RT-PCR is carried out using 5 _g of total RNA extracted from cultured endothelial cells. After denaturation in diethylpyrocarbonate-treated water for 10 minutes at 70_C, RNA is reverse-transcribed into first strand cDNA using SuperScriptII Rnase H- reverse transcriptase (10 units/reaction, Gibco BRL) and 0.5 _g of oligo (dT) as primer, at 42_C for 50 min in a total volume of 20 _l in a buffer containing (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTP, 10 mM dithiothreitol, 20 units Rnasin). Reverse transcriptase is inactivated at 70_C for 15 min and the RNA template was digested by Rnase H at 37_C for 20 min. Each experiment includes samples devoid of reverse transcriptase (negative controls) to exclude amplification from contaminating genomic DNA.

Semi-quantitative RT-PCR amplification is performed with a PTC 225 thermal cycler (MJ Research), following a 1 minute period of denaturation at 94_C, under the following conditions: denaturation at 94_C for 30 seconds, annealing at 55_C for 30 seconds, and extension at 72_C for 30 seconds, for a total of 30 cycles. The assay mixture contained 20 mM Tris HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 _M of oligonucleotide primers, dNTPs (100 _M of dATP, dGTP, dTTP, 10 _M dCTP), 0.5 _Ci of [^{32}P] dCTP, 0.5 units of Taq DNA polymerase, and 5 _l of a hundred fold diluted cDNA mixture. The final product is extended for 3 min at 72_C. In each experiment, RT positive controls (templates containing cDNA encoding for VEGF) and negative control (without DNA) are included.

The PCR products are then subjected to electrophoresis on 6% (w/v) acrylamide gels. Radioactivity in each band is quantified by the storage phosphorimaging technique. The screens are scanned using a Fuji BAS 2000. The signal is quantified in Photo Stimulating Luminescence (PSL) units using the Tina image analysis software. Results are expressed for each sample as band intensity relative to that of GAPDH. An optimum number of PCR cycles is determined in the region of exponential amplification. Logarithmic dilutions of the cDNA mixture are

used to verify the linear correlation between the intensity of the radioactive signal and the initial amount of cDNA.

VEGF ELISA

96-well plates coated with anti-human VEGF monoclonal antibody are purchased from R&D Systems (Mineapolis). HDMEC or hTERT(+)HDMEC culture supernatants are added into the wells and VEGF is bound by the immobilized antibody. After extensive washing, a peroxidase linked polyclonal antibody specific for VEGF is added to the wells; after washing, a peroxidase substrate solution is added and the plates were incubated for 5 minutes at room temperature. Optical density is mesured at 620 nm with an ELISA plate reader (BioRad).

B. VEGF Receptor Analysis

VEGF receptors, Flt-1/VEGFR1 and flk/KDR/VEGFR2 are analyzed by immunoprecipitation with anti-human Flt-1 and anti-KDR IgG (Santa Cruz BioTech, Santa Cruz, CA) according to standard procedures (Herron GS, Banda MJ, Clark EJ, et al. Secretion of metalloproteinase by stimulated capillary endothelial cells. II. Expression of collagenase and stromelysin activities is regulated by endogenous inhibitors. J. Biol. Chem 1986, 261:2814-2818). Phosphorylation of each receptor is assessed by immunoprecipitation followed by immunoblotting with murine anti-human phosphotyosine IgG (Upstate BioTech, New York, NY) according to the protocol described by (Kupprion C, Motamed K, Sage EH. SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. Journal of Biological Chemistry 1998, 273(45):29635-40). Data are normalized to total protein and blots are reprobed with beta actin (1:2000 in TBS/0.1% Tween 20/3% BSA for 2 hrs.; Boehringer Mannheim, Indianapolis, IN). Experiments are typically performed in duplicate on PD5 and PD25 primary HDMEC and PD30 and PD60-90 hTERT(+)HDMECs in defined growth media (EBM; Clonetics, San Diego, CA) at 90% confluency in the presence of 0.1-1.0nM VEGF (Pepro Tech).

C. NO Analysis

ecNOS transcripts, protein levels and concentration of total nitrogen oxides (NOx) are determined by semi-quantitative RT-PCR, western blotting and chemo-luminescence according to standard protocols essentially as described below. hTERT(+)HDMECs at PD30, 60 and 90 are compared to PD5 and PD25 of primary control EC at subconfluency (50-80%).

SDS-PAGE is performed using 8% separating gel according to previously published procedures (Chan, VT, Hultquist, K, Zhang, DN, Romero, LI, Lao, D and Herron, GS. Membrane type matrix metalloproteinase expression in human dermal microvascular endothelial cells. J.Invest Dermatol. 1998;111:1153-1159). 80-90% confluent HDMEC or hTERT(+)HDMEC at third passage (48hr after plating) are washed in cold PBS pH 7.4, and solubilized in 1 % SDS, 10 mM Tris pH 7.4. Cell lysates are boiled for 5 minutes and centrifuged at 2500xg for additional 5 minutes to remove insoluble material. Protein concentration is determined using the Bradford assay (BioRad). An equal amount of protein (17.5 g) is loaded into each lane, separated by SDS-PAGE, and transferred to nitrocellulose by electroblotting at 4_C. The nitrocellulose membrane is blocked in a solution containing 1% BSA, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20 at 4 _C overnight. After 3 washings of 10 min with PBS/1% Tween 20 the membrane is incubated with 1:250 dilution of the anti-human endothelial eNOS IgG in the blocking solution for 4 h at room temperature. Duplicate samples are also reacted with the anti-human smooth muscle actin IgG as controls. After decanting the primary antibody and washing as above, the membrane is incubated with a horseradish peroxidase labeled-sheep anti-mouse antibody for 1 hour at room temperature. The membrane is developed using the luminescent method of ECL (Amersham) after exposure to substrate for 5 minutes followed by visualization on X-ray film. The film is then photographed and digitally analyzed using the Electrophoresis Documentation and Analysis System 120 by Kodak according to the manufacture's instructions.

RT-PCR analysis for eNOS

Reverse transcriptase polymerase chain reaction (RT-PCR) is used to asssess eNOS mRNA expression by 80-90% confluent, normal, SSc, HDMEC, hTERT(+)HDMEC (at various passage numbers) and Loc Scl DMEC approximately 48hr after plating (2^{nd} to 3^{rd} passage). 10 ng total RNA, isolated with STAT-60 (Tel- Test "B" Inc., Friendswood, TX) is used as template for cDNA synthesis in a volume of 50 _l according to manufacture's recommendations (Invitrogen, San Diego, CA). For PCR amplification, 3 _l of cDNA is used as a template and amplification conditions are 95_C for 5 minutes followed by 95_C for 1 minute, 58_C for 1 minute and 72_C for 1 minute for 30 cycles in a Perkin Elmer Cetus 9600 thermal cycler. Amplification is performed in a total volume of 50 _l containing 1.5 mM MgCl2, 0.1 mM of each nucleotide, 5 pmol of each primer, and 2.5 U of Taq Polymerase (Perkin Elmer, Branchburg, NJ). To amplify the 400 bp eNOS cDNA fragment the upstream primer used is 5' GTG ATG GCG AAG CGA GTG AA 3' and the downstream primer is 5' CCG AGC CCG AAC ACA CAG AAC 3'. Replicate samples are used to amplify the 300bp GAPDH cDNA using upstream primer 5'GGG GAG CGA GAT CCC TCC AAA ATC AAG TGG GG and downstream primer 5'GGG TCA TGA GTC CTT CCA CGA TAC CAA AGT TG. The PCR products (10 _l) are analyzed on 1.5% agarose gel electrophoresis, stained with ethidium bromide, destained and photographed under ultraviolet light using the Electrophoresis Documentation and Analysis System 120 by Kodak according to the manufacture's instructions. Relative eNOS transcript levels are determined by comparing the ratio of eNOS:GAPDH densitometric units for HDMEC, hTERT(+)HDMEC (at various passage numbers) SSc, Loc Scl and control samples. Significant differences between samples are determined by ANOVA using the Statview SE+ program (Abacus Concepts Inc.). $p < 0.05$ was considered statistically significant.

Measurement of Cell Secreted Nitrogen Oxides

Second passage, PECAM(+) DMEC from 3 adult control, 3 SSc, HDMEC, and hTERT(+)HDMEC and 3 Loc Scl patients are plated in duplicate at 50%

confluency on gelatin-coated 35 mM petri dishes. At confluency (5 x 10^5 cells) Complete Media is removed and plain DMEM is substituted. One dish per sample is stimulated with $1x10^{-7}$ M calcium ionophore A23187 (Sigma) in plain DMEM. All media is collected at 16 hr, snap frozen and stored at -20 _C for measurement of nitrogen oxides (NOx).

NOx in cell media is measured using a chemiluminiscence apparatus (model 2108, Dasibi Corp., Glendale, CA) as previously described (Tsao PS, McEvoy LM, Drexler H, Butcher EC, Cooke JP; Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine, Circulation 1994 May;89(5):2176-82). An aliquot (50_l) of media is injected into boiling acidic vanadium (III) chloride. This technique utilizes acidic vanadium (III) chloride at 98_C to reduce both NO2 - and NO3- to NO, which is detected by the chemiluminescence apparatus after reacting with ozone. Signals from the detector are analyzed by computerized integration of curve areas. Standard curves for NaNO2/NaNO3 are linear over the range of 50 pM to 10 nM. NOx values are analyzed using the Anova Statview SE+ program as described above; $p<.0.05$ was considered statistically significant.

D. Pharmacologic Blockade of cNOS

To test the sensitivity of hTERT(+)HDMECs versus primary control HDMEC to NO inhibitors, cells are treated with a potent competitive inhibitor of cNOS, N-4-nitro-L-arginine (L-NNA) (Sigma, St. Louis, MO) at 0.1-2.5mM concentrations. Survival curves (MTT; Sigma, St. Louis, MO) are measured for the cell lines following L-NNA treatments at baseline and in the presence of apoptotic inducers (TNFα/AMD, LPS/CHX, UVC, SFM; Sigma, St. Louis, MO) and recombinant Endostatin. The survival curves are compared among hTERT(+)HDMECs and controls.

Example 3

Comparison of Levels of Activated Matrix Metalloproteinases and TIMPs

A. Evaluation of TSP-1

Experiments performed in support of the present invention have shown that TSP-1 was differentially expressed in HDMEC derived from pathologic skin samples (e.g. Junctional Epidermolysis Bullosa; JEB) vs neonatal HDMEC.. Immunofluorescence micrographs showed TSP-1 reactivity as wispy confluent deposits in control cell matrix, whereas JEB HDMEC retained very little cytoplasmic TSP-1 and no deposition into the matrix (photo-exposure time control TSP = 14 sec; photo-exposure for JEB TSP = 38 sec). Occasional JEB HDMEC stained weakly for TSP-1. TSP-1 levels are evaluated in the hTERT(+)HDMECs of the present invention versus primary controls using a combination of IF microscopy and RT-PCR.

B. Evaluation of ES

ES isoforms produced are evaluated for several hTERT-bearing cell lines compared to primary HDMEC controls at different PD using the same immunoblotting procedures described above for evaluation of ES.

C. Measurement of MMP Activities

Replicate hTERT(+)HDMECs (PD30, 60, 90) and primary controls (PD5, PD25) are seeded at equal densities, grown to confluence and switched to EBM media (Clonetics, San Diego, CA) containing growth factors, but no serum, for 72 hrs. Media is collected and total protein measured (Pierce, Rockford, IL). Zymography is performed according to previously published procedures (Herron GS, Banda MJ, Clark EJ, et al. Secretion of metalloproteinase by stimulated capillary endothelial cells. II. Expression of collagenase and stromelysin activities is regulated by endogenous inhibitors. J. Biol. Chem 1986, 261:2814-2818; Chan, VT, Hultquist, K, Zhang, DN, Romero, LI, Lao, D and Herron, GS. Membrane type matrix metalloproteinase expression in human dermal microvascular endothelial cells.

J.Invest Dermatol. 1998, 111:1153-1159). In addition, conditioned media are analyzed by a fluorescent substrate assay (FSA).

The FSA is an assay system for MMP activities based on recently-developed fluorogenic substrates that utilize 7-methoxycoumarin (MOC)-labeled MMP-specific small peptides ("Knight" substrate; Knight CG. Fluorimetric Assays of Proteolytic Enzymes. Met Enzymol 1995, 248:18-34). Release of MOC from the 2,4-dinitrophenyl-quenched peptide by active MMPs results in proportional increases in fluorescence with time. Activities of recombinant MMP-2 and MMP-9 using the Knight substrate were readily detectable and yielded linear initial reaction rates over 10 minutes. Calculated initial rates of hydrolysis of the Knight substrate (4 uM) by MMP-2 (1.2 nM), MMP-9 (1.2 nM), and MMP-2 (0.6 uM) plus MMP-9 (0.6 uM) were 571.1 units/min, 208.2 units/min, and 242 units/min, respectively.

Activity measurements of conditioned media (CM) from primary normal human (NHK) and immortalized keratinocyte (NIK) cultures showed low net MMP activity detectable only in PMA-treated NHK and only after APMA activation (10.1 units/min/ug total protein). Values for concentrated CM from HDMEC cultures +/- PMA considerably higher and exhibited a linear increase with increasing volume of sample. Activities were completely blocked by 1,10 phenanthraline and partially blocked by rTIMP-1. Reverse zymography confirmed the presence of TIMPs.

The FSA is a fast and reproducible method for quantifying net MMP activities in CM.. TIMPs decrease the sensitivity of this assay system. Accordingly, the assay system is used in combination with zymography and/or after removal of TIMPs with anti-TIMP affinity beads.

Example 4

Measurement of CDK Inhibitor Patterns

Cellular extracts are prepared, total protein measured (BCA, Pierce) and immunoblotting performed using anti-p53, p21 (Oncogene Research, Cambridge, MA), pRB, p16, p27 (Santa Cruz Biotechnology, Santa Cruz, CA) according to published protocols.

Confluent hTERT(+)HDMECs and late passage primary HDMEC are induced to undergo apoptosis by two different methods, serum starvation/growth factor withdrawal and TNFα + AMD (actinomycin D). Time course studies are performed to assess changes in expression patterns at early and late apoptotic induction time points. Further, the effects of exogenous VEGF stimulation on phosphorylation status of these cell cycle proteins will be evaluated.

Example 5

Superior Durability of Telomerase Expressed Human Dermal Microvascular Endothelial Cells

Advantages of the present invention

a. **Uniform cell populations:** Herein the present invention demonstrates that hTERT(+)HDMEC retain the phenotypic and functional characteristics of young primary cells 3-5 times longer than primary cells (Yang et al 1999 J. Biol. Chem. 274:37:26141-48). These cells can be greatly expanded in culture thus providing the first ever uniform mass cell culture for angiogenic assay systems. Because all cells in the culture were derived from the same tissue source, they reproducibly respond to angiogenic and angiostatic stimuli the same way from generation to generation. All cells that are not life-extended via hTERT expression are selected against by continuous passaging beyond senescence of primary cells and thus cannot variably affect cell-cell interactions or present differential responses to stimuli. The hTERT(+)HDMEC mass cultures can either be used as mixed populations or can be fractionated further into different subpopulations representing different anatomic locations within the original tissue (e.g. precapillary arterioles vs postcapillary venules) via FAC sorting (Fig 4). This provides a pure, uniform and immortal cell population for incorporation into angiogenic matrices *in vitro* and *in vivo*.

b. **Durability of hTERT(+)HDMEC in angiogenic matrices:** Our previously published results demonstrated that the relative survival advantage of hTERT(+)HDMEC vs primary HDMEC is based upon both life span extension and apoptotic resistance (Yang et al 1999 J. Biol. Chem. 274:37:26141-48). The latter

phenomenon was tested by multiple different methods using multiple different apoptotic inducers. However, one class of inducers that was not tested includes extracellular matrix components. In this invention we demonstrate that hTERT(+)HDMEC resist activation of the apoptotic pathway induced by 3D collagen *in vitro*, a potent and relevant agent (Fig 14). This robust effect is highly reproducible and consistent with our published results with other inducers. It represents a hurdling the most important barrier to successful human microvascular remodeling assay system development. Taking this to the next level, we then tested the survivability of hTERT(+)HDMEC *in vivo* and demonstrated their durability was equivalent to or greater than young primary HDMEC (see below).

c. **Fluorescent-labeled hTERT(+)HDMEC:** Expression of marker enzymes (e.g. β–galactosidase) or fluorescent proteins (e.g. enhanced green fluorescent protein, eGFP) in primary MEC has rarely been reported due to low gene transduction efficiencies, inability to select and low survival rates. In this invention, we show that retroviral-mediated expression of eGFP in primary HDMEC results in high transduction efficiencies (Fig1, 2). These parental cells (GN1) were then "telomerized" with hTERT, continuously passaged and FAC-sorted by FITC to create a pure population of eGFP-expressing immortalized HDMEC (GNMEC1). This line reproducibly responds to angiogenic and angiostatic agents in the same manner as early passage parental primary cells and forms the basis of novel *in vitro* and *in vivo* microvascular remodeling assay systems.

d. ***In Vitro* angiogenic assay system:** Figure 15 demonstrates the ability of GNMEC1 to form "angiogenic webs" in response to plating onto a permissive matrix (e.g. Matrigel). Figure 16 demonstrates the superiority and versatility of using GNMEC1 to form fluorescent vascular structures in 3D Matrigel *in vitro* versus both senescent or young primary parental cells (GN1). To show the utility of this *in vitro* system, we pretreated GNMEC1 with two different cyclo-oxygenase (COX) antagonsists which are known to block angiogenesis (Jones et al. 1999 Nature Medicine 5:12;1418-23) and then plated the cells on Matrigel. Figure 17 shows a dose-response curve of angiogenic web blockade by indomethacin and NS-398 to

demonstrate the use of hTERT(+)HDMEC to screen therapeutic compounds for their potential efficacy in modulating blood vessel growth. The "tubulogenic process" can be followed and quantified utilizing cell lines such as GNMEC1 and digitally-converted fluorescence microscopic images of replicate cultures. Commercially available programs in current use with other cellular applications which maybe used to perform this process include MetaMorph® (Universal Imaging Corporation®, West Chester, PA) and the high-throughput imaging systems of Cellomics, Inc. (Pittsburgh, Pennsylvania).

e. ***In Vivo* human microvascular remodeling system:** In the present invention proof of principle is demonstrated indicating that hTERT(+)HDMEC can form human blood vessels in *in vivo*. SCID mice were implanted with Matrigel mixtures containing either b-FGF, primary HDMEC (PD10) or hTERT(+)HDMEC via subcutaneous injection on ventral thoracic surfaces. Implants were harvested at 2 weeks, sectioned and stained with H&E and anti-human basement membrane Type 4 collagen. As shown in Figure 18, host microvasculture is readily apparent invading matrices containing b-FGF alone but the absence of Type 4 collagen reactivity confirms specificity of murine vs human basement membranes. Immunoreactive lumenal structures in implants containing either primary or hTERT(+)HDMEC demonstrate formation of human microvasculature within the implants. The presence of red blood cells within these human vessels indicates host vasculature has anastamosed with the human vessels creating murine-human chimeric microvasculature.

To prove that the cells we implanted were responsible for the anti-human Type 4 collagen immunoreactivity, we implanted both GN1 (mid passage parental primary HDMEC) and GNMEC1 in the same SCID system as genetic-tagged cells for rapid identification. Thick sections of implants viewed by UV light demonstrate numerous fluorescent microvessels (Fig 19) proving that the origin of the vessels were human and demonstrating the utility of using eGFP-labeled hTERT(+)HDMEC in this assay system.

The superior durability of hTERT(+)HDMEC is demonstrated in Figures 19-20 in which increased survival of human microvasculature *in vivo* is apparent versus both mid passage and presenescent primary HDMEC. Quantitative comparison of vessel density using anti-human Type 4 collagen immuno-micromorphometry shows statistically significant and dramatic differences in survival characteristics of hTERT(+)HDMEC with time after implantation *in vivo*(Fig 21).

Proof of specificity is demonstrated by substitution of endothelial cells with either human dermal fibroblasts, human fibrosarcoma cells (HT-1080) or human embryonic kidney tumor cells (293) in Matrigel implants. Absence of Type 4 collagen immunoreactivity in these implants is shown in Figure 22. EGFP-labeled HT1080 and 293 cells demonstrates fluorescent tumor masses in the implant but absence of fluorescent microvasculature (Fig 23).

To demonstrate the potential of the *in vivo* system for testing different agents for their angiogenic and angiostatic qualities we pretreated GNMEC1 with either PMA, b-FGF, VEGF or anti-vitronectin receptor antibody (LM609). Figures 24-27 demonstrate variable effects of these agents on human microvessel formation *in vivo*. The greatest stimulatory effect is observed with b-FGF, whereas, both PMA and LM609 show angiostatic qualities (Fig 27). All agents were incorporated into Matrigel by mixing cells and agents together before implantation. The effect of continuous exposure at different concentrations was not performed but would be likely to greatly accentuate the results shown in these preliminary studies.

Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

What is claimed is:

1. A composition of endothelial cells, comprising immortal microvascular endothelial cells, said cells each comprising a recombinant expression cassette encoding telomerase, wherein said cells (a) have a normal karyotype, (b) are resistant to apoptosis relative to primary microvascular endothelial cells, and (c) are not transformed.

2. The composition of claim 1, wherein said cells are human dermal microvascular endothelial cells.

3. The composition of claim 1, wherein said telomerase is a human telomerase reverse transcriptase catalytic subunit.

4. The composition of claim 1, wherein said cells express one or more phenotypic traits expressed uniquely by young primary microvascular endothelial cells.

5. The composition of claim 4, wherein said phenotypic trait is selected from the group consisting of: surface receptors; signaling pathways; and both.

6. The composition of claim 1, wherein said cells stably express a transformed genetic marker.

7. The composition of claim 6, wherein said transformed genetic marker is enhanced green fluorescent protein (eGFP).

8. The composition of any one of claims 1 to 7, wherein said cells form human microvascular structures *in vitro*.

9. The composition of claim 8, wherein said human microvascular structures are quantifiable with digital imaging.

10. The composition of claim 9, wherein said digital imaging is fluorescence digital imaging.

11. The composition of any one of claims 1 to 7, wherein said cells demonstrate an extension of cellular life span and resistance to apoptosis comparable to young primary human dermal microvascular endothelial cells.

12. The composition of claim 11, wherein said cells demonstrate said extended cellular life span and resistance to apoptosis *in vivo* using a SCID-Human Chimeric Microvascular Remodeling Assay System.

13. A method of producing a composition of endothelial cells, comprising immortal microvascular endothelial cells, wherein said cells each comprise a recombinant expression cassette encoding telomerase, wherein said cells (a) have a normal karyotype, (b) are resistant to apoptosis relative to primary microvascular endothelial cells, and (c) are not transformed, comprising introducing said recombinant expression cassette encoding telomerase into human dermal microvascular endothelial cells and expressing said telomerase.

14. A composition produced by the method of claim 13, wherein said microvascular cells form neovasculature; wherein said host blood is transmitted through said neovasculature.

15. A composition produced by the method of claim 13, wherein said microvascular cells form neovasculature *in vivo*; wherein said host blood is transmitted through said neovasculature.

16. The composition of claim 12, wherein said microvascular cells form neovasculature *in vivo*; wherein said host blood is transmitted through said neovasculature.

17. A composition comprising microvascular cells; wherein said cells form neovasculature; wherein said host blood is transmitted through said neovasculature.

18. The composition of claim 17, wherein said cells comprise a genetic marker, wherein said marker is expressible in said cells; and wherein said marker is introduced into said cells through a molecule of recombinant DNA.

19. The composition of claim 18, further comprising microvascular cells; wherein said cells form neovasculature; wherein said host blood is transmitted through said neovasculature; and wherein.

20. The composition of any one of claims 14 to 19, wherein said neovasculature is human and wherein said *in vivo* host is non-human.

21. A composition of claim 20, wherein said *in vivo* host is a non-human mammal.

22. The composition of any one of claims 14 to 21, wherein said neovasculature is human and wherein said *in vivo* host is non-human; and wherein said cells analyzed with fluorescence digital imaging demonstrate said neovasculature is human and has characteristics that distinguish said neovasculature from non-human host.

23. A method that demonstrates neovasculature formed *in vivo* has characteristics that distinguish said neovasculature from *in vivo* host, comprising producing a composition of endothelial cells according to claim 13;

expressing in said cells a transformed genetic marker detectable by a digital imaging system; and

analyzing said cells with a digital imaging system so as to detect said genetic marker.

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24. The method of claim 23, wherein said digital imaging system is a fluorescence digital imaging system and said genetic marker is enhanced green fluorescent protein (eGFP).

ABSTRACT

The present invention relates to immortal microvascular endothelial cells having normal karyotype that demonstrate resistance to apoptosis, methods for producing said cells, and methods of use thereof.

Fig. 1A *Fig.1C* *Fig. 1E*

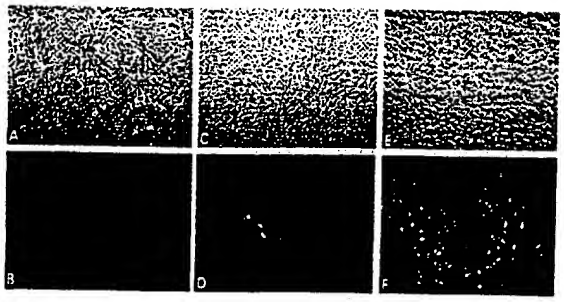


Fig. 1B *Fig. 1D* *Fig. 1F*

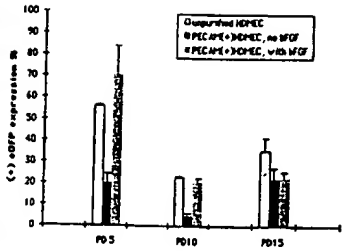


Fig. 2

Fig. 3A

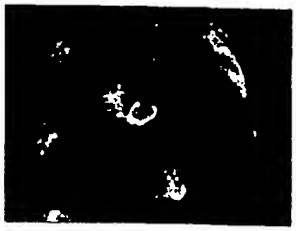


Fig. 3B

Fig. 4A

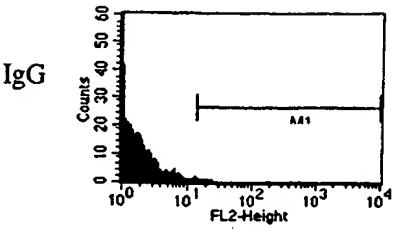
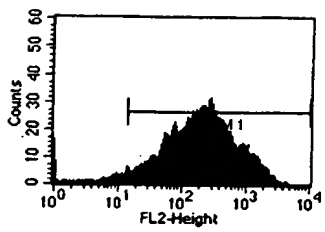


Fig. 4B



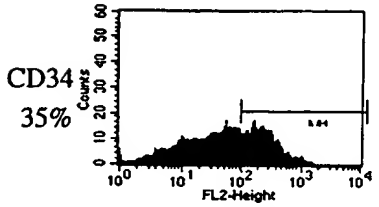


Fig. 4C

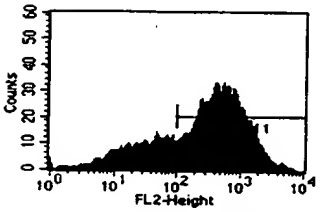


Fig. 4D

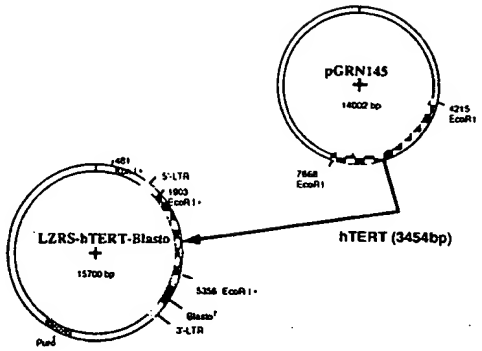


Fig. 5

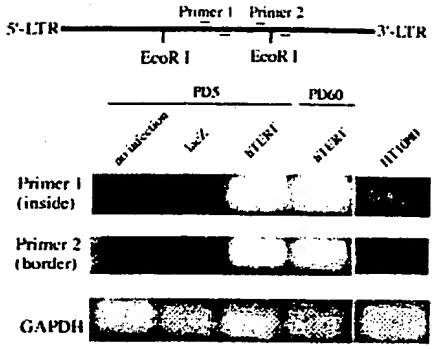

Primer 1
Primer 2
5'-LTR
3'-LTR
EcoR I
EcoR I
PD5
PD60
no infection
LacZ
hTERT
hTERT
HT1080
Primer 1 (inside)
Primer 2 (border)
GAPDH

Fig. 6

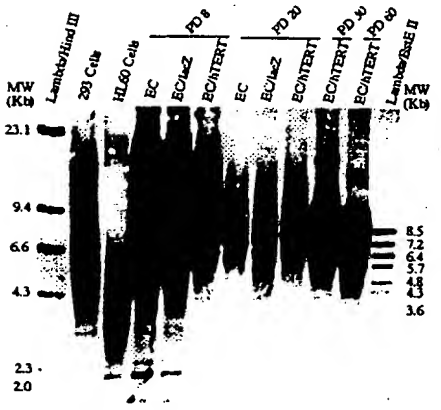

MW (Kb)
Lambda/Hind III
293 Cells
HL60 Cells
PD 8
PD 20
EC
EC/Luc2
EC/hTERT
EC
EC/Luc2
EC/hTERT
EC/hTERT
EC/hTERT
Lambda/BstE II
MW (Kb)
23.1
9.4
6.6
4.3
2.3
2.0
8.5
7.2
6.4
5.7
4.8
4.3
3.6

Fig. 7

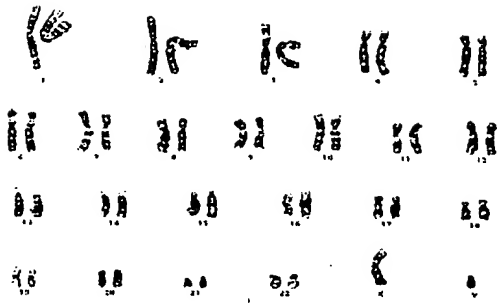


Fig.8

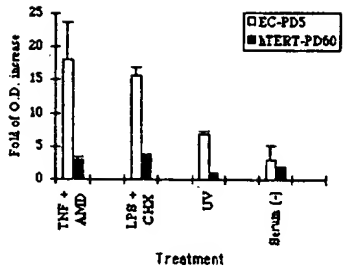


Fig. 9A

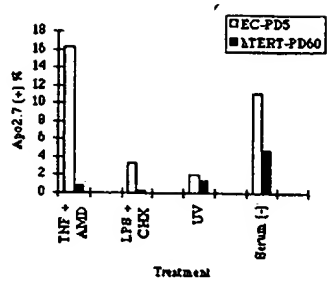


Fig. 9B

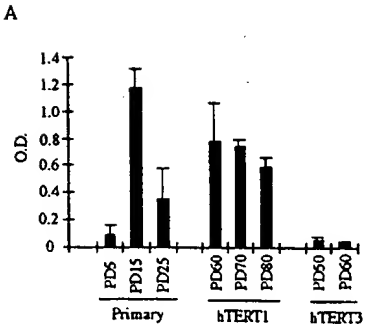


Fig. 10A

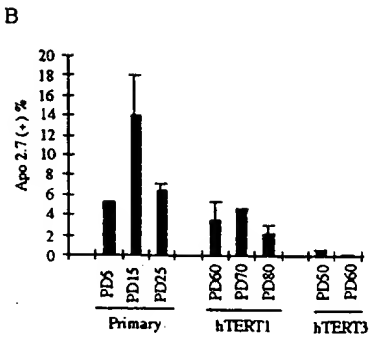


Fig. 10B

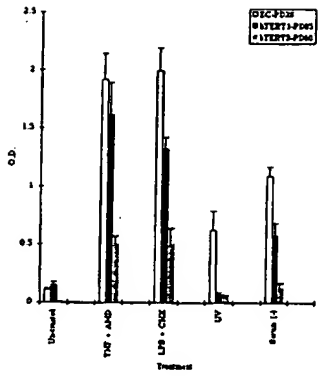


Fig. 11A

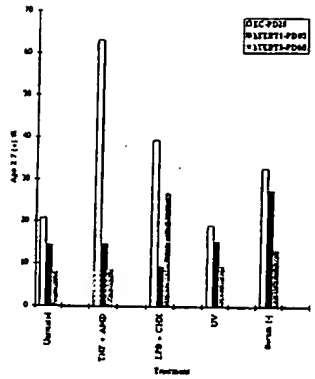


Fig. 11B

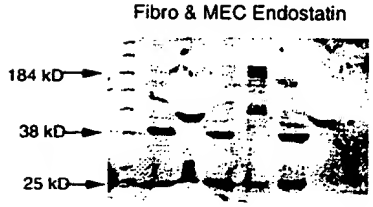


Fig. 12

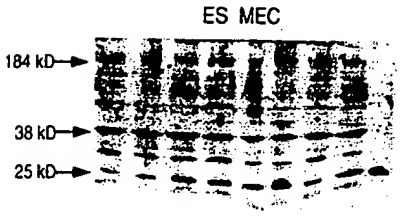


Fig. 13

Figure 14

Time course of apoptotic induction by 3D collagen overlay *in vitro* and superior survival of Tert-EC versus primary parental EC. By 16hr after collagen overlay, parental cultures are undergoing dissolution and apoptosis whereas, Tert-EC are still forming tubule structures. At 24hr, all parental cells are dead and Tert-EC are stable.

GN1PD32: eGFP-labeled primary HDMEC at PD32 (mid-late passage)

GNMEC1(+)PD40: hTERT(+) GNMEC-1 at PD40

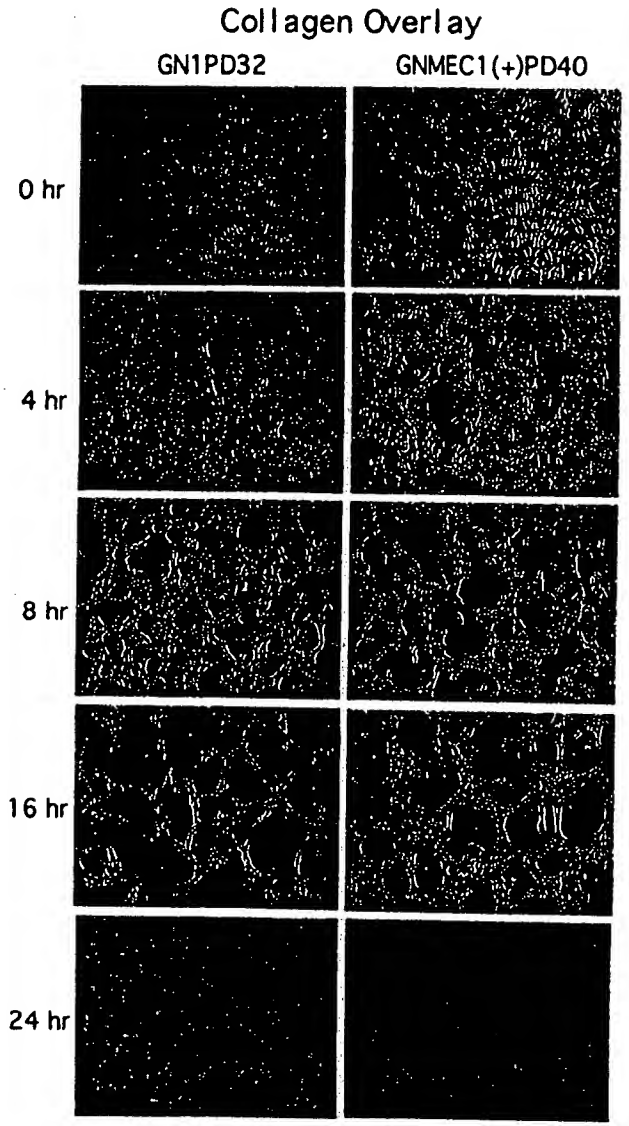


Figure 15

Time Course of High Density Matrigel Tubule Formation

4 Hr **8 Hr** **24 Hr**

Demonstration of the utility of using eGFP-labeled Tert-EC for tracking morphogenetic patterns of cells forming microvascular structures *in vitro*

Figure 16

Superiority and utility of eGFP-labeled Tert-EC in formation of vascular structures in 3D Matrigel *in vitro*.

Senescent parental EC (EC-PD38) show no vessels in 3D Matrigel compared to younger cells (EC-PD20). Vessels were numerous and prominently labeled in all Tert-EC cultures (hTERT1-PD56).

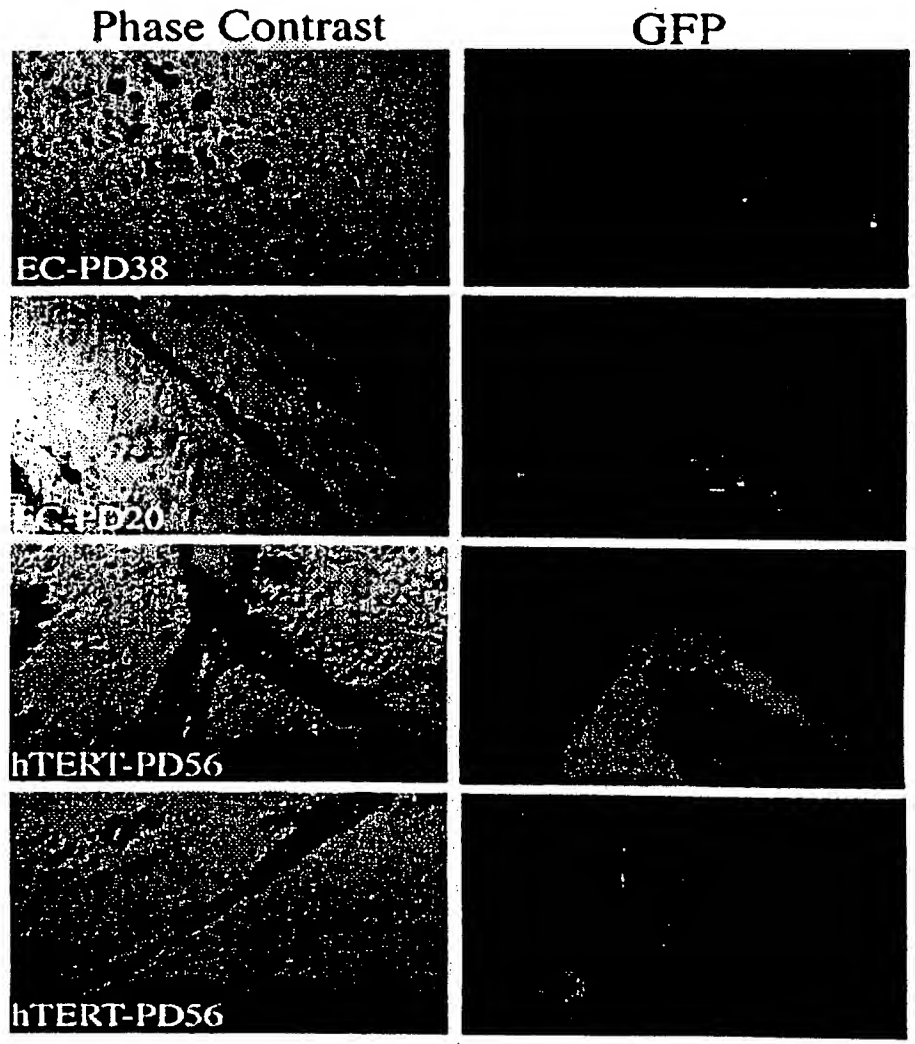


Figure 17

Inhibition of *in vitro* angiogenic web formation by cyclo-oxygenase (COX) antagonists

Tert-EC were incubated in the presence of two different COX inhibitors (indomethacin and NS-398) at different concentrations and examined 12hr after plating on Matrigel. Controls show no vessels in absence of Matrigel and numerous vessels in absence of or at low dose COX blockers. Graded inhibition of vessel formation is observed with increasing doses of COX blockers, with the COX-2 specific inhibitor (NS-398) 10-fold more potent than the general inhibitor, indomethacin.

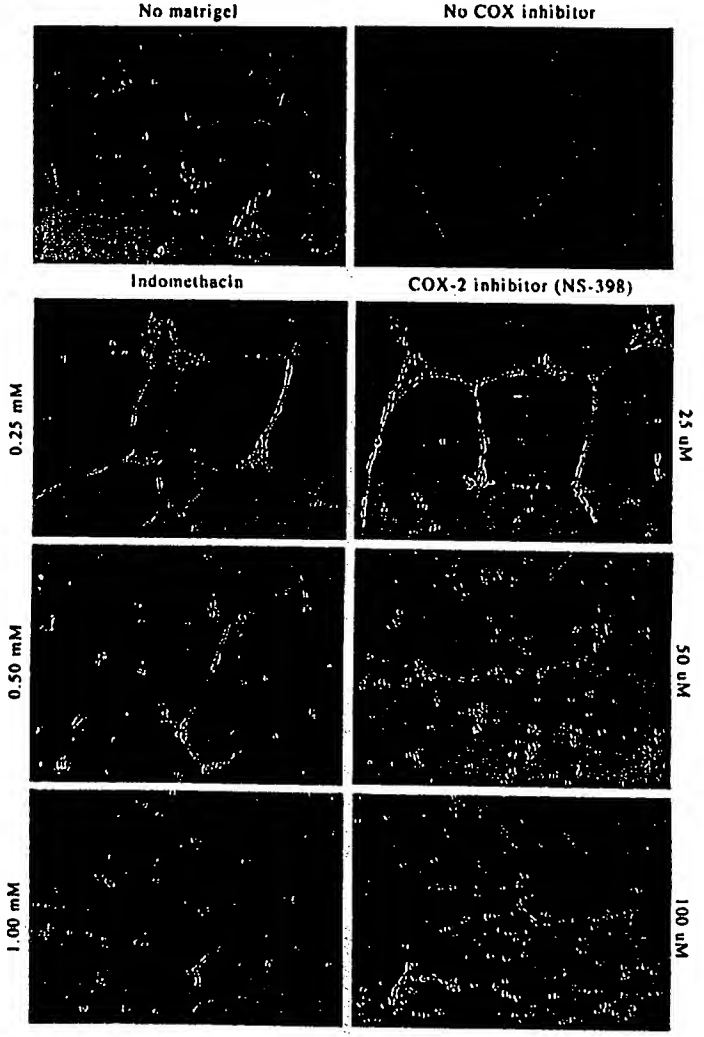


Figure 18

Tissue sections stained with H&E (A,D,G) and for anti-human Type 4 collagen (C,F,I) demonstrate the presence of host murine vessels in Matrigel implants containing bFGF (arrows) but absence of human basement membrane collagen (C). Both primary and Tert-EC demonstrate Col-4(+) vessels containing RBCs (arrows). IgG controls shows no IF reactivity (B,E,H). Results indicate human vessels have formed anastamoses with host mouse vessels (i.e. "chimeric" vessels).

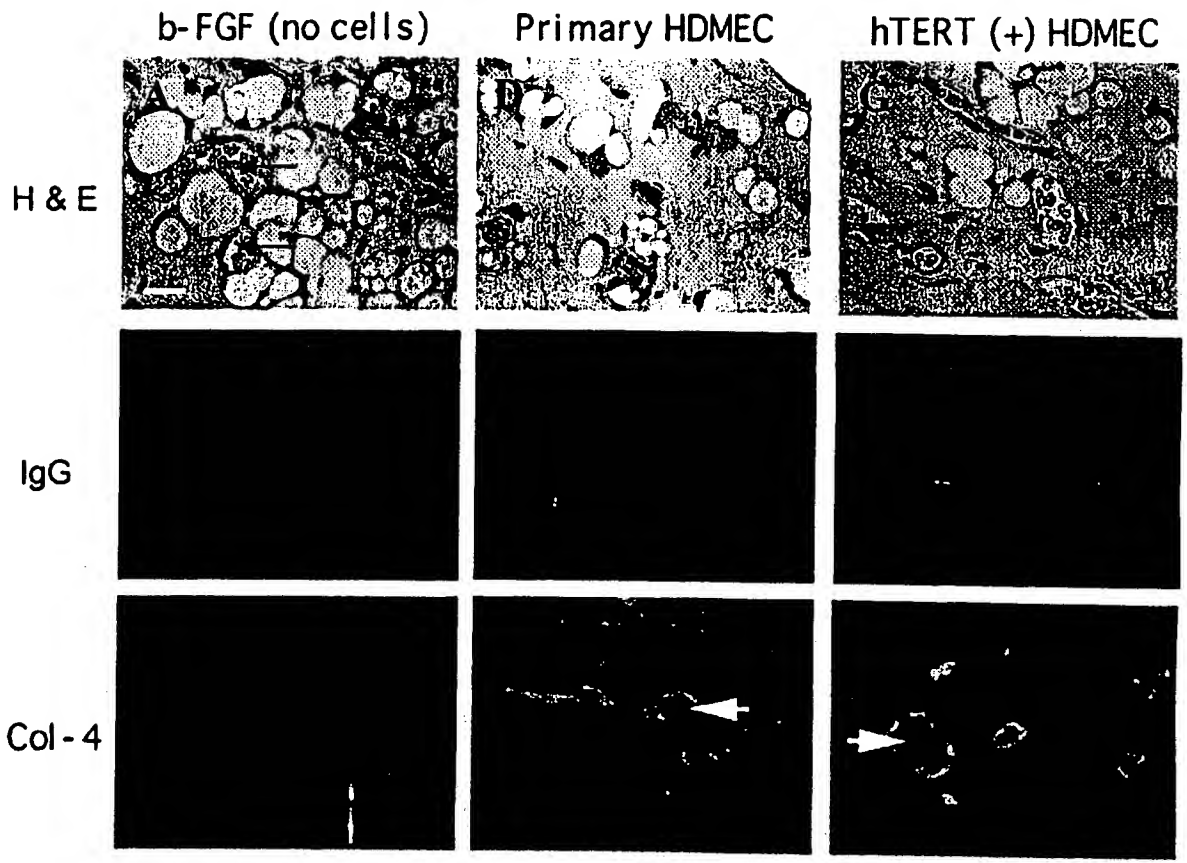


Figure 19 Superiority of Tert-EC at In Vivo Microvessel Formation

Early-mid passage parental eGFP-labeled HDMEC (GN1-PD20) were directly compared to Tert-EC line (GNMEC-1) for their ability to form fluorescent microvessels *in vivo* using the SCID mouse Matrigel implantation assay system. TertEC demonstrated more fluorescent vascular structures at all time points after implantation versus parental line.

Figure 20 Superiority of Tert-EC at In Vivo Microvessel Formation

Use of Type 4 collagen IF reactivity to quantify human microvessels *in vivo* demonstrates superior durability of Tert-EC (GNMEC1) versus late passage parental cells (GN1PD34). Younger parental cells (PD20) show many small diameter vessels.

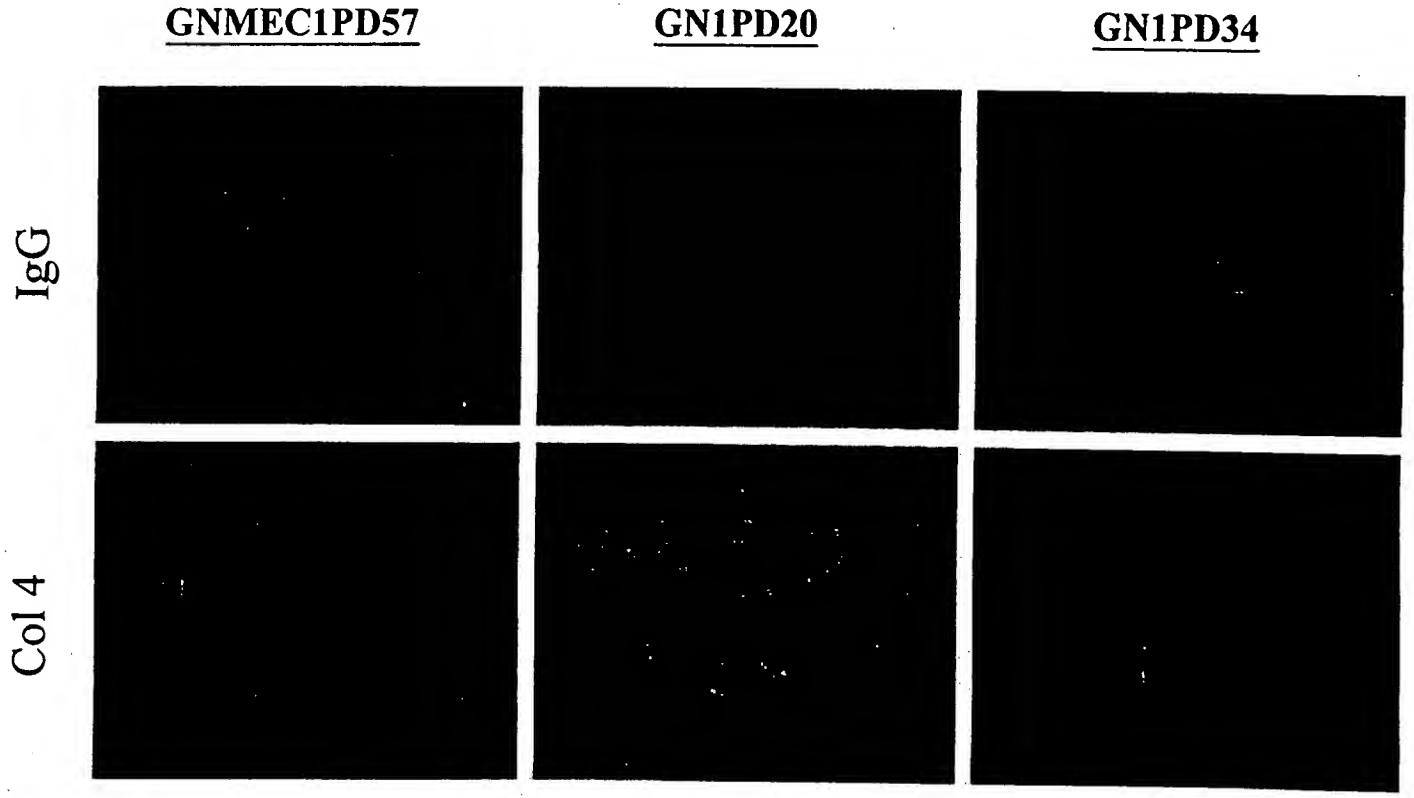


Figure 21 Superiority of Tert-EC at In Vivo Microvessel Formation

Quantitative comparison of microvessel density by IFmicromorphometry using anti-human Type 4 collagen demonstrates equal number of vessels in young primary (GN1PD12) vs Tert-EC (GNMEC1) and maintenance of vessels at 2, 4 and 6 weeks after implantation in Tert-EC (>25); whereas, mid and late passage parental EC show both decreased numbers and loss of vessels with time *in vivo*. Dermal fibroblasts (Fb) and human fibrosarcoma cells (HT-1080) show no vessel formation (see Figures 22, 23).

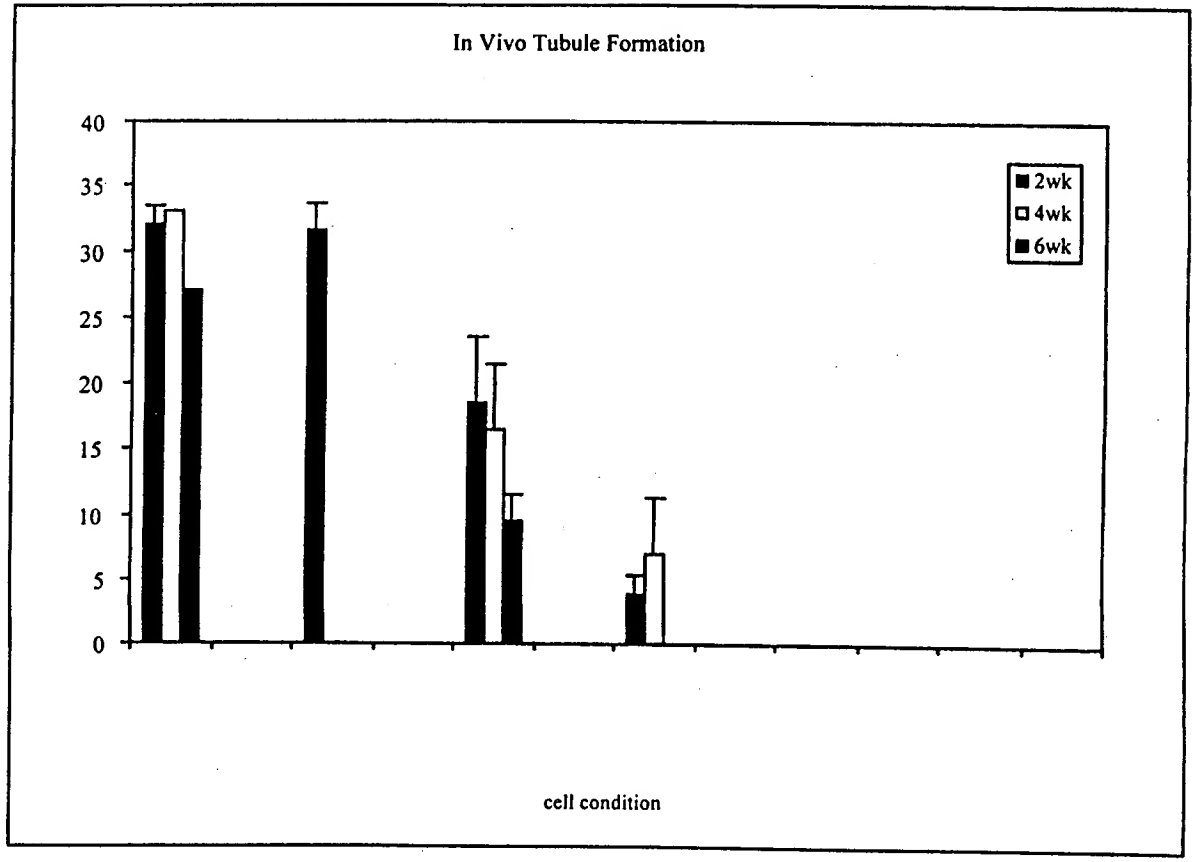


Figure 22

Tissue sections stained with H&E (A,D,G) and for anti-human Type 4 collagen (C,F,I) demonstrate the presence of human vessels in Matrigel implants containingTert-EC (G,I) but absence of human basement membrane collagen (C,F) in implants containing HT1080 cells or human dermal fibroblasts. IgG controls shows no IF reactivity (B,E,H). Results indicate lack of human vessel formation in implants containing nonECtypes

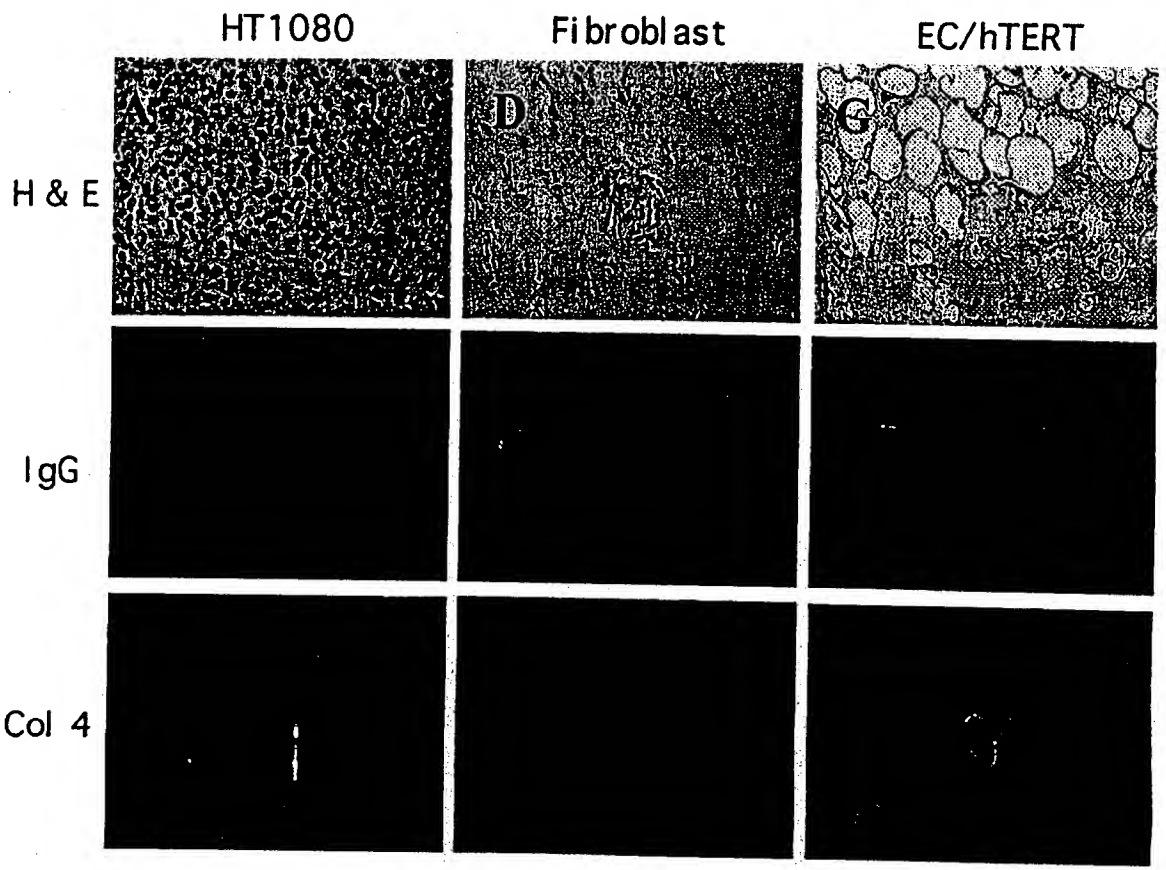
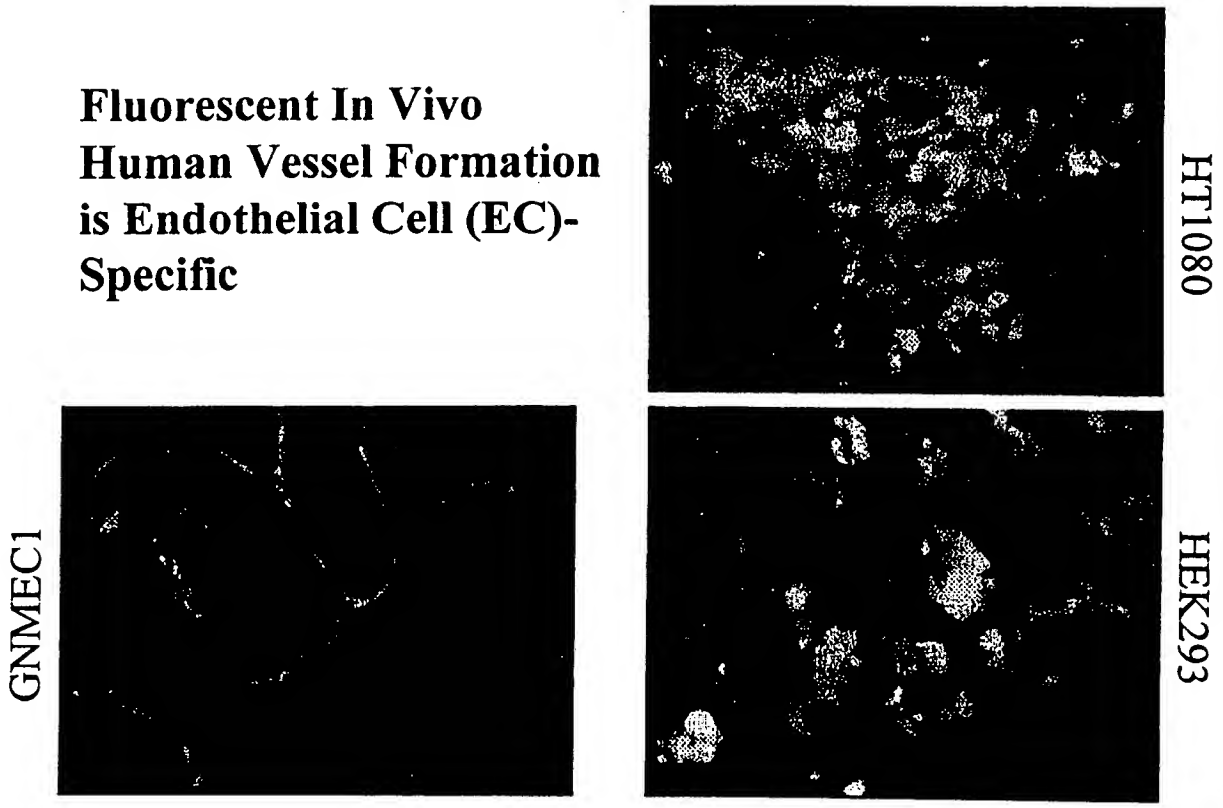
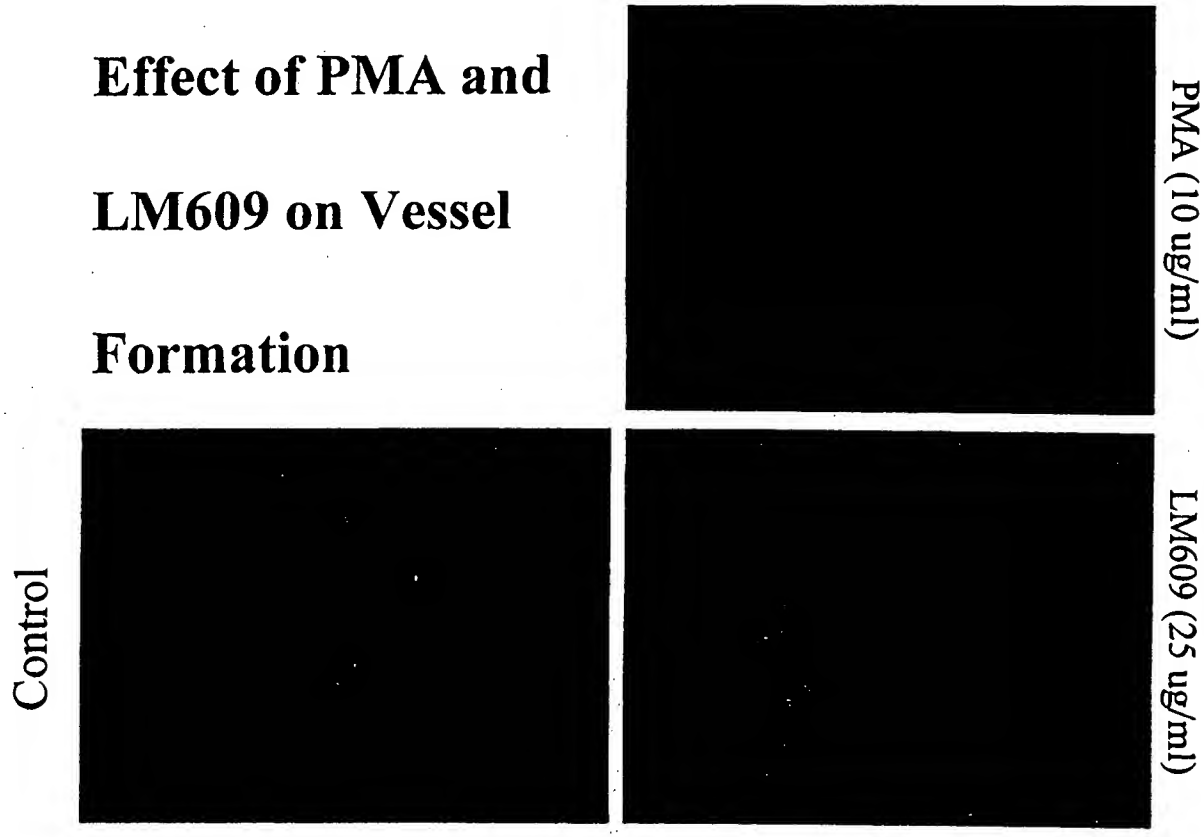


Figure 23



Human fibrosarcoma cells (HT1080) and human embryonic kidney tumor cells (HEK293) expressing eGFP show fluorescent tumor masses but no microvessels, whereas, Tert-EC (GNMEC1) form obvious fluorescent vessels.

Figure 24



Treatment of Tert-EC with phorbol ester (PMA) or vitronectin receptor ($\alpha_v\beta_3$) -antagonist (LM609) for 2 hrs prior to implantation *in vivo*. Results show PMA has a slight negative effect on vessel density, whereas, LM609 appears to increase vessels relative to control, untreated cells.

Figure 25

Effect of PMA and LM609 on *In Vivo* Vessel Formation

Quantification of vessel density by IF staining of human Type 4 collagen shows effects of PMA and LM609, as described in Figure 24.

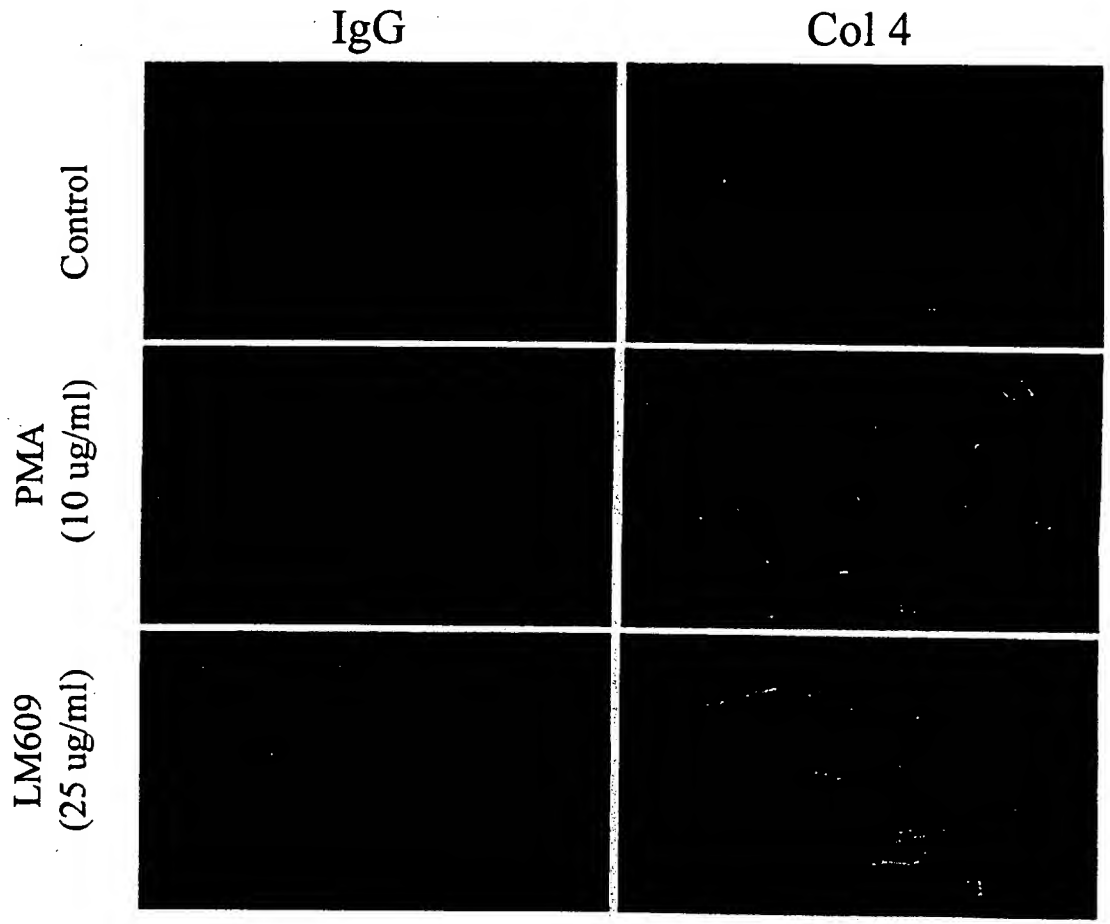


Figure 26

Quantification of vessel density by IF staining of human Type 4 collagen shows effects of b-FGF and VEGF. Results indicate FGF increases vessel density, whereas, VEGF has little effect.

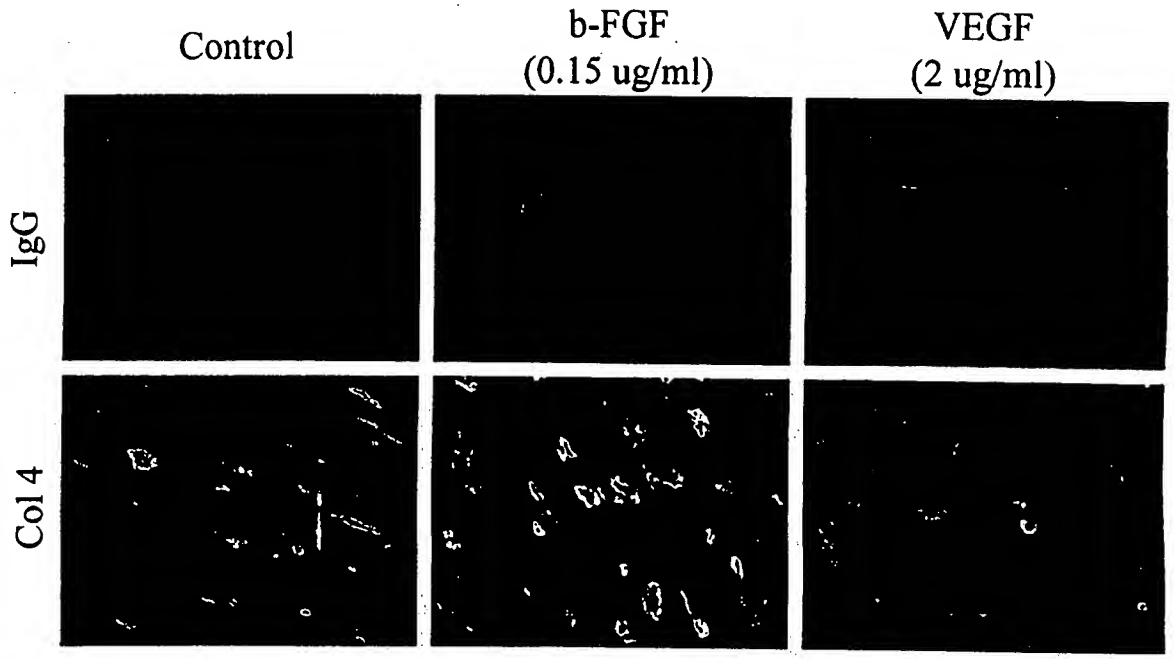
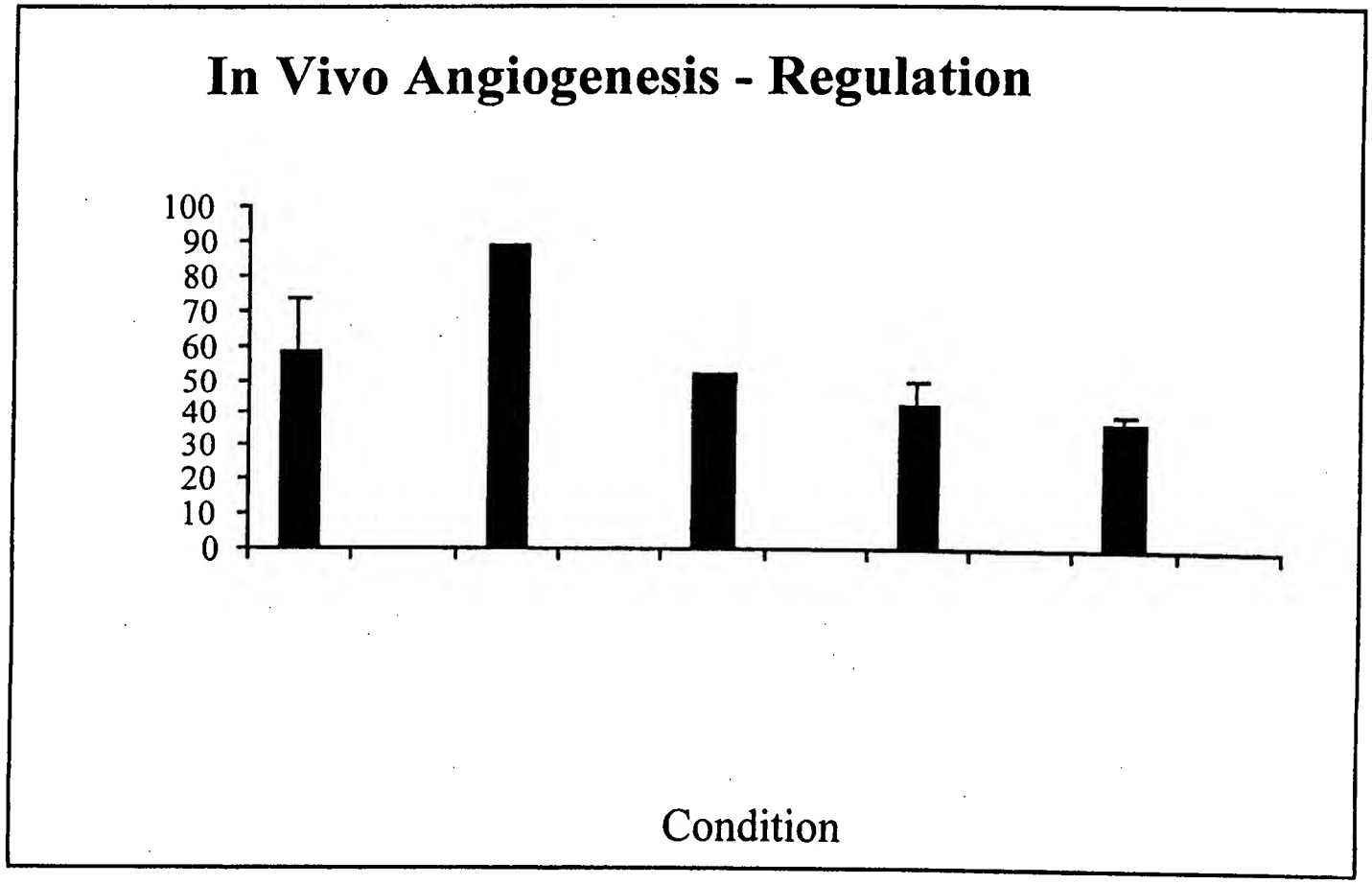


Figure 27



Effect of different angiogenic regulators on GNMEC1 *in vivo* microvessel formation. Results show significant changes in vessel density only by b-FGF.